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Directed Evolution of LuxI for Enhanced OHHL Production

Pavan Kumar Reddy Kambam, Daniel J. Sayut, Yan Niu, Dawn T. Eriksen, Lianhong Sun

Department of Chemical Engineering, University of Massachusetts Amherst, 686 North Pleasant Street, Amherst, Massachusetts 01003; telephone: +1-1-413-545-6143; fax: +1-1-413-5451647; e-mail: lsun@ecs.umass.edu

Received 2 November 2007; revision received 6 March 2008; accepted 11 March 2008

Published online 19 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21901

ABSTRACT: Quorum sensing is a common mechanism used by bacteria to coordinate population behavior, and is involved in a variety of biological processes, such as bioluminescence, virulence factor synthesis, antibiotic production, and biofilm formation. To engineer the LuxI enzyme of the LuxI–LuxR quorum-sensing system, we developed a high throughput genetic selection to identify LuxI mutants with improved OHHL (3-oxo-hexanoyl homoserine lactone) synthesis in *E. coli*. Using this genetic selection, we created LuxI mutants with improved OHHL synthesis rates and yields through directed evolution, identifying three LuxI mutants after two generations. An in vivo semi-quantitative method allowed for verification of the genetic screen and OHHL yields were quantified using HPLC–MS/MS, revealing an 80-fold increase in a mutant culture compared to the wildtype culture. In addition to OHHL, the yields of C6HSL (hexanoyl homoserine lactone) and C8HSL (octanoyl homoserine lactone) were also improved, and a slight change in substrate specificity towards C6HSL production was observed. Based on alignment with the crystal structure of EsaI, a homolog of LuxI, two mutations are most likely involved in enhancing the interactions between the enzyme and the substrates. The high throughput genetic selection and the semi-quantitative method can be conveniently modified for the directed evolution of LuxI homologs. The identification of these LuxI mutants has implications in synthetic biology, where they can be used for the construction of artificial genetic circuits. In addition, development of drugs that specifically target quorum sensing to attenuate the pathogenesis of gram-negative infectious bacteria might also benefit from the insights into the molecular mechanism of quorum sensing revealed by the amino acid substitutions.

Biotechnol. Bioeng. 2008;xxx: xxx–xxx.

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KEYWORDS: directed evolution; genetic selection; quorum sensing; synthetic biology

Yan Niu's current address is Department of Biochemistry, Purdue University, 175 S. University Street, West Lafayette, IN 47906.
Correspondence to: L. Sun
Contract grant sponsor: NSF CAREER Award
Contract grant number: CBET 0747728

Introduction

Quorum sensing is an intercellular communication mechanism used by bacteria to synchronize population behavior (Miller and Bassler, 2001). The LuxI–LuxR quorum sensing circuit present in the marine bacterium *Vibrio fischeri* is composed of a signal molecule synthase, LuxI, that synthesizes the diffusible signal molecule OHHL (Fig. 1), and a transcription factor, LuxR, that activates gene expression from the P_{luxI} promoter when the local accumulation of OHHL exceeds a threshold concentration (Fuqua et al., 2001). Quorum-sensing systems composed of LuxI and LuxR homologs have been identified in many gram-negative bacteria, with each system using distinct acyl-homoserine lactone signaling molecules that vary in the degree of oxidation and length of the acyl side chain (Waters and Bassler, 2005). Although the biological function of quorum sensing remains controversial (Hense et al., 2007), it plays a significant role in many biological processes, including virulence factor synthesis, biofilm formation, and plasmid conjugation (Fuqua and Greenberg, 2002). In particular, it is well-documented that quorum sensing is involved in many infectious diseases (de Kievit and Iglewski, 2000; Fux et al., 2005). For example, the LasI–LasR and RhlI–RhlR quorum-sensing systems in the human pathogen *P. aeruginosa* regulate the expression of more than 150 genes, a significant portion of which are involved in pathogenesis and biofilm formation (Nouwens et al., 2003; Schuster et al., 2003). Disruption of the quorum-sensing systems makes *P. aeruginosa* more susceptible to antibiotics and the immune-response (Wu et al., 2001).

Discovery of the natural products that inhibit quorum sensing and subsequent examinations in a mouse model indicate that drugs that specifically target quorum sensing might represent a novel class of antibiotics effective in treating bacterial infection (Baveja et al., 2004; Tang et al., 1996). As such, understanding the molecular mechanism of quorum sensing could provide insights into relevant infection mechanisms, and facilitate the development of quorum-sensing based anti-bacterial drugs (Taha et al.,

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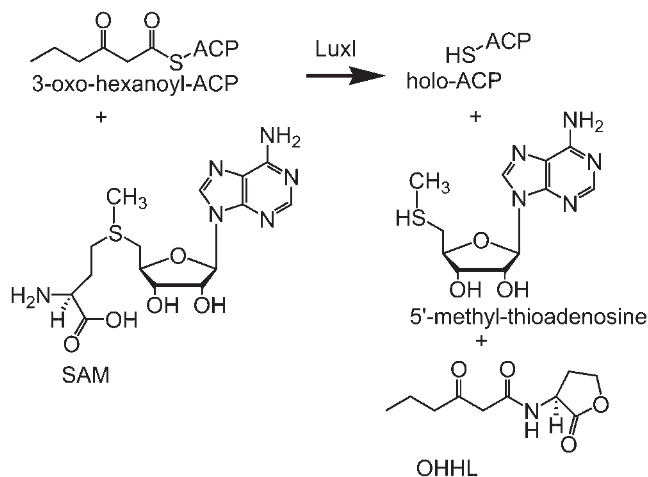

Figure 1. Reaction catalyzed by LuxI, an AHL synthase.

2006). It is also likely that such drugs would have broad-spectrum antibacterial activity, as quorum sensing is present in a large variety of bacteria (Harraghy et al., 2007; McCormack, 2006; Rasmussen and Givskov, 2006). Because quorum sensing inhibitors do not interfere with the basic life cycle of bacteria, no harsh selection pressure is introduced (Bjarnsholt and Givskov, 2007). Consequently, using quorum sensing inhibitors could significantly reduce the development of bacterial drug resistance, which has been an increasing challenge for infectious disease control (Stewart and Costerton, 2001).

Quorum sensing has been used in the construction of a variety of engineered biological systems as a mechanism to control gene regulation, and has been successfully implemented in bacterial, eukaryotic, plant, and mammalian cells (Neddermann et al., 2003; Weber et al., 2005; You et al., 2006). These systems generally utilize the unique capability of quorum sensing to regulate gene expression in a cell-density dependent manner to construct artificial genetic circuits that are capable of regulating the complicated dynamic behaviors of a cell population (Basu et al., 2005; You et al., 2004). As quorum-sensing systems are self-sufficient, no chemical inducers or temperature changes are required to activate the systems. Consequently, quorum sensing allows for completely autonomous activation of gene expression. This property is desired in large-scale fermentations to achieve improved production of pharmaceuticals or therapeutic proteins (Sayut et al., 2007a), and artificial quorum sensing systems in *E. coli* and yeast *S. cerevisiae* have been developed to meet applications in industrial biotechnology (Bulter et al., 2004; Chen and Weiss, 2005).

The diverse applications of quorum sensing will benefit from engineered systems with altered responses (Yokobayashi et al., 2003). Engineering of LuxI, LuxR and their homologs will provide insight into the molecular interactions of the components, which are valuable for the design of drugs that target quorum sensing (Rasmussen and Givskov, 2006). Engineered quorum-sensing systems can also be used to construct artificial genetic circuits and complex regulatory cascades that will help improve the understanding of natural biological networks and allow for the improvement or creation of novel system properties (Andrianantoandro et al., 2006). In this regard, LuxR has been engineered both to enhance response to OHHL and to alter its substrate specificity (Collins et al., 2006; Sayut et al., 2006, 2007b). Similarly, LuxI can be engineered for enhanced activity to further increase the applications of quorum sensing. Site-directed mutagenesis has been used to identify amino acid residues essential for LuxI function (Hanzelka et al., 1997), but LuxI mutants with increased OHHL production have never been reported. In this report, we described the engineering of LuxI for enhanced OHHL synthesis via directed evolution, and the generation of LuxI mutants with significant increase in OHHL production.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions

E. coli strain Top10F′ was used for all cloning and expression experiments. Cells were cultured in Luria-Bertani (LB) medium at 37°C for liquid cultures (shaken at 225 rpm) or grown on agar plates. All cultures contained kanamycin (50 μg/mL), chloramphenicol (100 μg/mL) and varied concentrations of ampicillin depending on the selection conditions. For the semi-quantitative analysis experiments, cultures were grown in 250 mL of M9 medium supplemented with 10% LB medium at 30°C with a shaking speed of 180 rpm. All cultures were incubated with 1 mM of IPTG unless otherwise indicated. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated.

Plasmid Construction

Plasmids plux-GFPuv and plac-LuxRI were obtained from the Arnold Group at Caltech (Collins et al., 2005). All the restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Plasmid pLuxIR was constructed from plac-LuxRI by first replacing the *luxR* gene with a *luxI* gene, yielding plasmid pLuxII. Subsequent substitution of the second *luxI* gene with a *luxR* gene generated plasmid pLuxIR. Plasmid plux-Amp was constructed by substituting the *plux-gfpuv* fragment of the plasmid plux-GFPuv with a *plux-kan* fragment constructed previously, followed by replacement of *kan* gene with an *amp* gene. The *plux-kan* fragment was created by fusing the P_{luxI} promoter with a kanamycin resistance gene using SOEing (Splicing by Overlap Extension) PCR (Horton, 1995). Plasmids used in the experiments were shown in Figure 2.

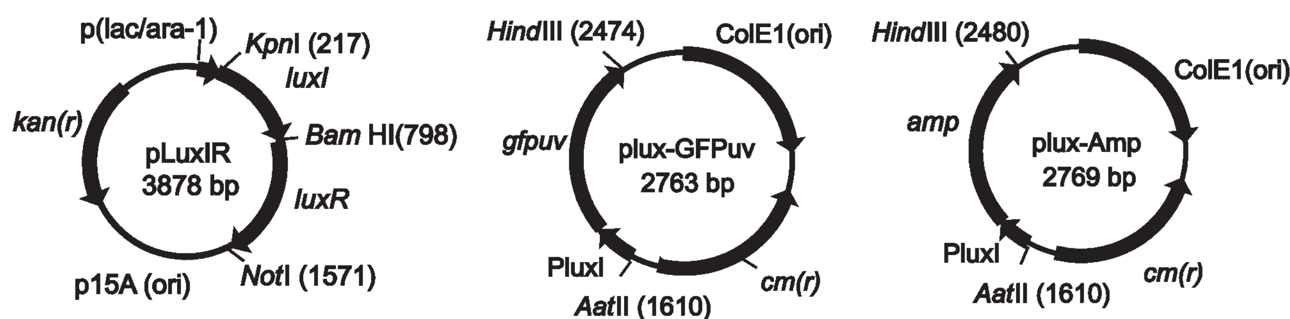

Figure 2. Plasmids used in the research.

Directed Evolution of LuxI

The random mutagenesis library of LuxI was generated by error-prone PCR (EP-PCR) using *Taq* DNA polymerase. The PCR conditions were similar to those used previously for creation of LuxR mutant libraries (Sayut et al., 2006). Specifically, the PCR mixture includes 7 mM of $MgCl_2$, 0.3 mM of $MnCl_2$, 10 mM Tris–HCl, 50 nM KCl buffer (pH 8.5 at 25°C), ~30 ng plasmid DNA as the template, 30 pmol of each primer, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, and 5 U *Taq* polymerase in a total volume of 100 μL. The primers LuxIF (5′-ATAGGTACC-ATGACTATAATGATAAA-3′) and LuxIR (5′-ATAGGAT-CCTTAATTTAAGACTGCTT-3′) were used to amplify the LuxI gene. The PCR reactions were carried out in a thermal cycler (PTC-200, MJ Research) with 30 cycles of the following conditions: 94°C for 60 s, 55°C for 60 s and 72°C for 110 s. PCR products were purified using a QiaGen PCR purification kit. The libraries were constructed by ligating *Kpn*I- and *Bam*HI-digested pLuxIR with the purified PCR fragments using T4 DNA ligase. The ligation mixture was then transformed into Top10F′ competent cells containing plux-Amp plasmid by electroporation (Gene Pulser, BioRad, Hercules, CA). The transformants were grown on agar plates with 1,000 μg/mL (first round) or 1,400 μg/mL (second round) ampicillin to select mutants with enhanced activities.

In Vivo Quantification

Colonies identified from the library were grown overnight and purified to recover the pLuxIR and plux-Amp plasmid mixture. Mutant pLuxIR was isolated from the mixture by selectively digesting plasmid plux-Amp in the mixture using *Nhe*I and *Pvu*I restriction enzyme, and transforming the resulting mixture into Top10F′ electroporation competent cells. Liquid cultures grown from single colonies were used to purify the pLuxIR plasmid. The recovered pLuxIR plasmid was co-transformed with plux-GFPuv for use in the in vivo semi-quantitative method. 0.5 mL of the overnight cell cultures harboring wildtype or mutant LuxI enzymes were inoculated into M9 medium with 10% LB medium (250 mL), and grown for 10 h. Four independent cultures of the mutant and wildtype LuxI enzymes were grown and samples were used to measure fluorescence intensity and cell density. Cultures of the wildtype LuxI without induction and cultures containing the fluorescence protein but not LuxI were used as references. After an initial 2-h growth, samples were collected every 30 min to determine cell densities and fluorescence intensities. Cell density was determined by measuring the absorbance at 595 nm using a spectrophotometer (UV1101, Biotech Photometer, Cambridge, UK). Fluorescent measurements were taken using a microtiter plate reader (SPECTRAmax GEMINI Xs, Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths of the plate reader were set at 395 and 509 nm, respectively.

Extraction of OHHL

Plasmid pLuxIR containing a wildtype or mutant LuxI gene was transformed into *E. coli* TOP10F′. A single colony was inoculated into 3 mL of LB medium, and 0.5 mL of the overnight cultures was inoculated into 500 mL of M9 medium with 10% LB medium. Cell cultures were pelletted by centrifuging cultures at 3,000*g* for 20 min at 4°C after grown for 10 h to reach the OD_{595} 1.2. The supernatant was extracted twice with 250 mL of dichloromethane. The organic phase was then dried over anhydrous magnesium sulfate to remove all traces of water, followed by filtration and vacuum evaporation to remove all organic solvent. The residue was dissolved in 1.5 mL of water, filtered, and stored at −20°C for future analysis. Approximately 20% of the total OHHL in the culture was recovered by this procedure, which is a much lower efficiency than what has been reported for similar procedures (Makemson et al., 2006). Data were reported as $\bar{x} \pm t_{\alpha,f} s/\sqrt{n}$, where $\bar{x}$ is the mean value, $t_{\alpha,f}$ is the *t*-table value with a confidence level of $(1-\alpha)$ and a degree of freedom of $f = n-1$, and n is the number of measurements. We use a confidence level of 0.9 for all the data.

Reverse-Phase HPLC–MS/MS

An Agilent Technologies Series 1100 vacuum degasser, LC pump, autosampler, and a C18 reversed-phase column (210×2.1 mm, 5 μm) were used for isolation of OHHL. The column was equilibrated for 15 min with methanol (30% in water, v/v), after which 80 μL of each stored sample was injected for analysis. The injected samples were eluted with methanol (30% in water, v/v) for 5 min followed by an isocratic elution with 95% methanol for 15 min. For analysis of C6HSL and C8HSL, the samples were eluted with 60% of methanol for 5 min followed by an isocratic elution with 95% methanol for 15 min. The eluted samples were injected into a spectrometer directly via a split without introducing any additives.

The eluted samples were analyzed by ion trap mass spectrometry using a Bruker Esquire-LC spectrometer. The parameters used for the mass spectrometer have been reported previously (Morin et al., 2003). Particularly, the positive ion mode was used to isolate OHHL (m/z 214), C6HSL (m/z 200), and C8HSL (m/z 228) for MS–MS fragmentation. Extracted ion chromatograms (m/z 102, corresponding to the protonated homoserine lactone ring) were used for quantifying OHHL, C6HSL, and C8HSL. Peak areas were calculated by integrating the peaks using DataAnalysis 3.0 (Bruker Daltonics, Bremen, Germany). Standards of OHHL, C6HSL, and C8HSL were used to determine the retention time of each compound, which were consistent with the retention times of samples. Samples were also spiked with standards to verify the retention times. Standard curves for OHHL, C6HSL, and C8HSL were prepared by using standard solutions. Each sample was analyzed three times to determine reproducibility. Samples of high concentrations were then spiked with standard solution of known concentrations, and the concentrations of the AHLs of the resulting samples were analyzed by the same HPLC–MS/MS procedure. The total concentration of each AHL was consistent with the calculated concentration, indicating the matrix effect was negligible.

Calibration curves determined by known concentrations of OHHL, C6HSL, and C8HSL were used to calculate the concentrations of the injected samples. To obtain highly linear responses, two calibration curves were used for concentrations of OHHL ranging from 10 to 50 and 50 to 450 μM, respectively. Three calibration curves were used for concentrations of C6HSL and C8HSL ranging from 2 to 10, 10 to 50, and 50 to 350 μM, respectively. Coefficients of determination (R^2) of all fitted straight lines were equal to or larger than 0.99. Errors were reported in the same manner as described in the previous section.

Western Blotting

To prepare *c-myc*-tagged LuxI proteins, a wildtype or mutant LuxI gene was cloned into the plasmid pBAD/myc-His A (Invitrogen, Carlsbad, CA) using primers LuxIF (*Nco*I) (ATACCATGGATACTATAATGATAAA) and LuxIR (*Hin*dIII) (ATAAAGCTTATTTAAGACTGCTTT). The resulting plasmid was transformed into Top10F′ using electroporation, and a single colony was inoculated into 3 mL LB medium and the culture was grown overnight at 37°C. Twenty microliters of the overnight culture was inoculated into fresh 3 mL LB medium, and the sample was grown for 3 h at 30°C, followed by introduction of 0.2% arabinose to induce gene expression for additional 4 h. Cells were collected by spinning down at 13,200 rpm and the cell pellet was resuspended with 250 μL of 1× fast break lysis buffer (Promega, Madison, WI), 1 mM PMSF and 10 μL of 20 mg/mL lysozyme (Fischer Scientific, Houston, TX). After incubated for 20 min, 1 μL DNase (Promega) was added and the sample was incubated for additional 5 min. Cell debris was then removed by centrifuging at 13,200 rpm for 10 min, and the resulting supernatant was mixed with an equal volume of SDS sample buffer followed by boiling for 3 min. The sample was then centrifuged for 10 min at 13,200 rpm, and the proteins were fractioned by standard SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane (Amersham, Piscataway, NJ) using electrophoresis (BioRad Trans-Blot Cell apparatus). The membrane was incubated with 10 mL blocking buffer PBST (PBS with 0.05% tween-20 and 5% nonfat dry milk) for 1 h, and then was washed twice with PBST followed by an overnight incubation with 10 mL blocking buffer containing 2 μL anti-*Myc*-His HRP antibody (1:5,000 dilution rate). The membrane was washed twice with PBST and chemiluminescence was developed using an ECL detection kit (Amersham) and recorded on a film (Krackeler, Albany, NY). The film was then scanned and the image was analyzed by Image J software.

Results

Genetic Selection

In order to use directed evolution on LuxI for enhanced OHHL synthesis, we developed a high throughput genetic selection to efficiently identify LuxI mutants with the desired property. The genetic selection was based on a genetic screen that has been used previously for the directed evolution of LuxR (Collins et al., 2005; Sayut et al., 2006). The genetic selection was established using the pLuxIR and plux-Amp plasmids, and a similar strategy has been used to develop a genetic selection for the directed evolution of LuxR for increased specificity (Collins et al., 2006). Plasmid pLuxIR consists of the *luxI* and *luxR* genes under control of a $P_{lac/ara}$ promoter. Plasmid plux-Amp carries a P_{luxI} regulated β-lactamase gene (*amp*) that provides ampicillin resistance. When both plasmids are co-transformed into *E. coli*, OHHL synthesized by LuxI interacts with LuxR to activate β-lactamase expression from the P_{luxI} promoter, allowing the cells to proliferate in the presence of certain concentrations of ampicillin.

Validation of the genetic selection is shown in Figure 3. Cells harboring both plasmids with the wildtype LuxI were

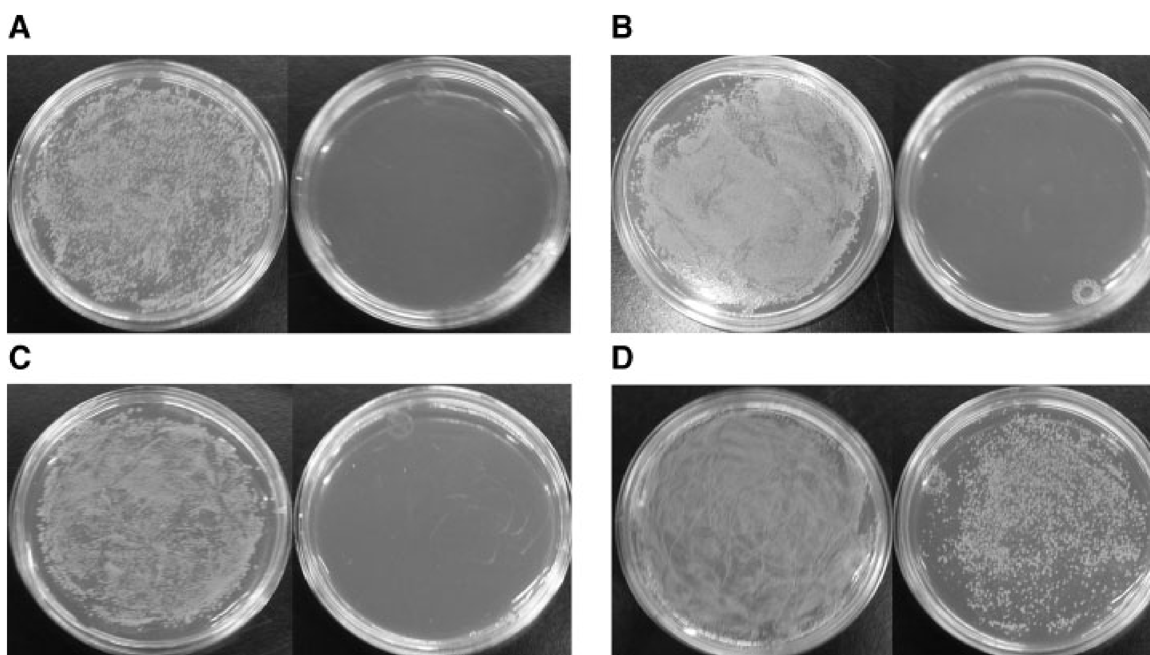

Figure 3. Validation of the genetic selection. **A**: Plates with 100 or 500 μg/mL ampicillin (left to right). **B**: Plates with IPTG (1 mM) and 100 or 500 μg/mL ampicillin (left to right). **C**: Plates with OHHL (200 nM) and 100 or 500 μg/mL ampicillin (left to right). **D**: Plates with IPTG (1 mM), OHHL (200 nM) and 100 or 500 μg/mL ampicillin (left to right).

able to grow at ampicillin concentrations up to 500 μg/mL. Above 500 μg/mL, no wildtype cells grew independent of the IPTG concentrations used to induce LuxI and LuxR expression. This indicates that the quantity of OHHL synthesized by the wildtype LuxI is inadequate to produce sufficient β-lactamase from the P_{luxI} promoter to rescue cells in the presence of 500 μg/mL of ampicillin. Therefore, 500 μg/mL ampicillin was used as the minimal concentration for growth inhibition of the transformed cells (Fig. 3A). To verify that cells could be rescued by increased OHHL production, exogenous OHHL was added to the plates. OHHL addition rescued the cells on plates with IPTG (Fig. 3D) but not on plates without IPTG (Fig. 3C), indicating that the expression level of LuxR induced by IPTG was sufficient to activate the P_{luxI} promoter in the presence of adequate amounts of OHHL. Overall, these results demonstrated that LuxI mutants with increased activities could be selected using the genetic selection, and the minimal selection conditions were established for the first round of directed evolution experiments.

Directed Evolution of LuxI and Characterizations of the Mutants

After verifying the genetic selection, we then used it for the directed evolution of LuxI for enhanced OHHL synthesis. Random mutagenesis libraries of LuxI were generated by error-prone polymerase chain reaction (EP-PCR) (Cadwell and Joyce, 1994). The mutant transformants were incubated on agar plates with 1,000 μg/mL ampicillin in the presence of IPTG, a concentration much higher than the minimal ampicillin concentration for growth inhibition of the wildtype cells. Three mutants were selected and recovered for further verification. In order to efficiently verify mutant activity, we developed a semi-quantitative in vivo method to measure the activity of the LuxI mutants relative to the wildtype (Fig. 4). By using P_{luxI} to regulate a green fluorescent protein (GFPuv), cells expressing LuxI and LuxR will develop fluorescence when the population exceeds a certain density. LuxI mutants with increased OHHL productions activate the P_{luxI} promoter at lower cell densities, resulting in more intense fluorescence when compared to cells expressing the wildtype LuxI at the identical cell densities. Fluorescence measurements of growing cultures were taken at regular time intervals over a period of 10 h. As shown in Figure 5A, significant fluorescence was observed for cells expressing mutant 1A at an OD_{595} of 0.15, while cells expressing the wildtype LuxI developed significant fluorescence at an OD_{595} of 0.3, indicating that mutant 1A exhibited enhanced OHHL synthesis in vivo. In addition, the cells expressing mutant 1A had higher fluorescence intensities than cells expressing the wildtype LuxI until OD_{595} 1.0. No significant difference in growth rates was observed for cells with the mutant or wildtype LuxI. The two other identified mutants did not show any increased fluorescence in comparison to the wild type (data not shown).

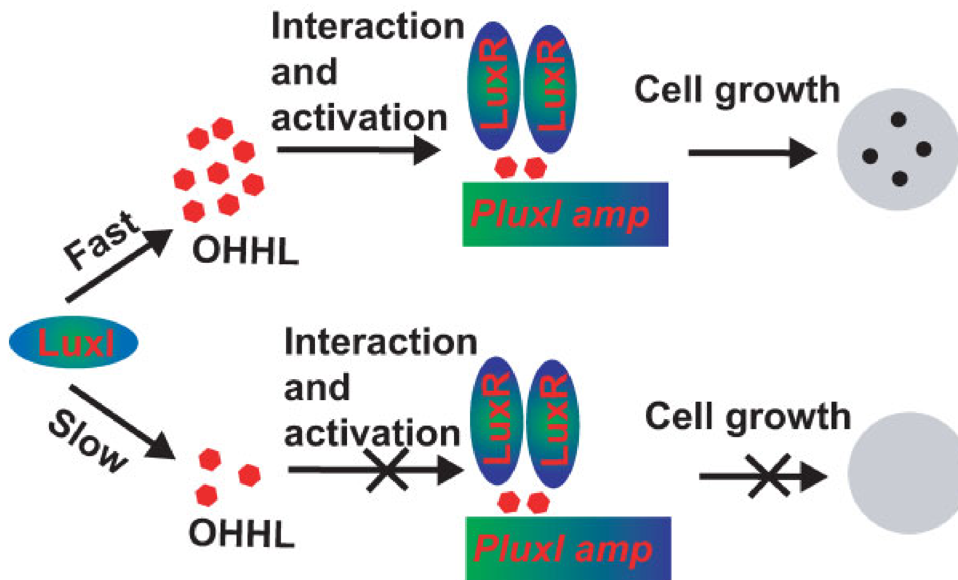

Figure 4. Principle of the genetic selection. An active LuxI enzyme produces sufficient amount of OHHL to form LuxR-OHHL complexes to activate the expression of β-lactamase to rescue cells, while a less active LuxI enzyme fails to produce adequent amount of β-lactamase to rescue cells in the same time period. In the in vivo semi-quantification method, a GFPuv rather than β-lactamase is used, resulting in development of fluorescence of cell cultures.

LuxI uses SAM and 3-oxo-hexanoyl-ACP as the substrates to synthesize OHHL (Fig. 1). Because 3-oxo-hexanoyl-ACP protein complex is not readily available, determining the kinetic parameters of the mutant or wildtype LuxI is difficult. As a result, we characterized mutant LuxI enzymes by measuring the total OHHL yields of the cell cultures. Using HPLC–MS/MS, the enhanced OHHL synthesis of mutant 1A was verified by determining its relative OHHL

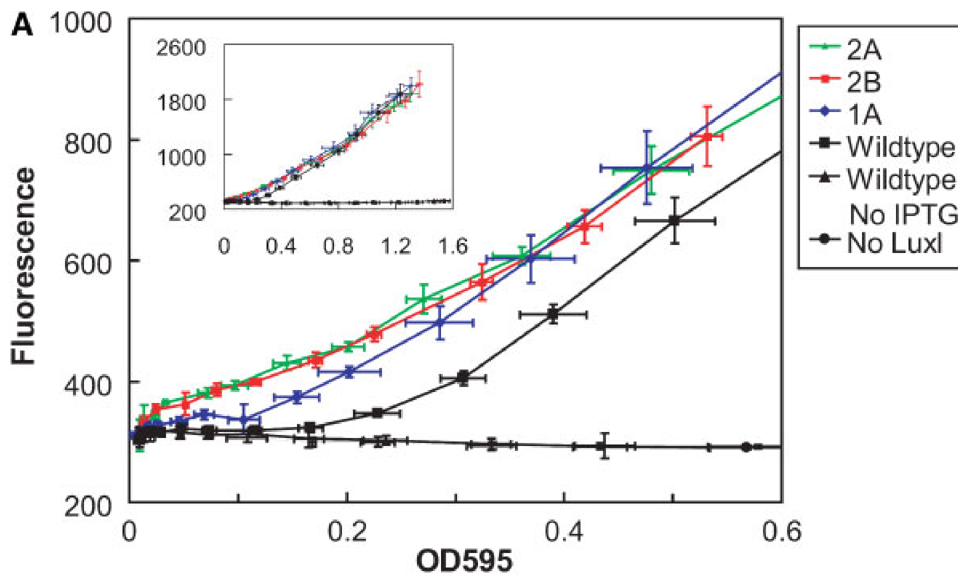

Figure 5. **A**: In vivo semi-quantification of LuxI mutants. A wildtype LuxI culture without induction by IPTG and a culture containing GFPuv but not LuxI were used as references. The inset shows the responses over the whole OD range measured. **B**: Concentrations of OHHL, C6HSL, and C8HSL in extracted samples from cells expressing mutant and wildtype LuxI measured by HPLC–MS/MS. The samples were prepared by extracting AHLs from 500 mL of cell cultures and concentrating to a final volume of 1.5 mL in water. **C**: Expression levels of the wildtype and mutant LuxI determined by Western blotting. Mutant 2A and 2B exhibit approximately twofold increase in the expression level than the wildtype LuxI.

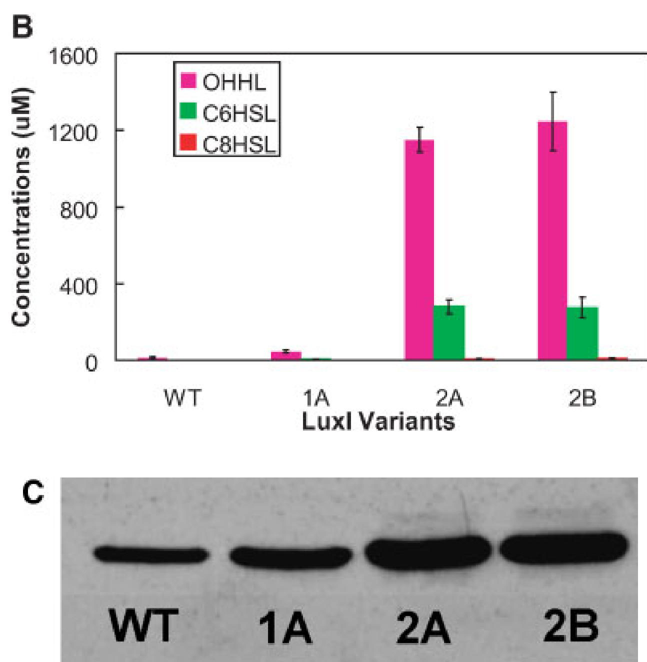

Figure 5. (*Continued*)

yield (Morin et al., 2003). The OHHL in the culture was recovered by extraction and concentrated prior to analysis. As shown in Figure 3C, the concentration of OHHL extracted from cells expressing mutant 1A was more than threefold higher than that from cells expressing the wildtype LuxI. This result confirmed that the in vivo semi-quantitative method could be used to verify LuxI mutants identified from the genetic selection.

Using mutant 1A as the template, we performed a second round of directed evolution. Two mutants (mutant 2A and 2B) were identified from the EP-PCR library by growth on selection plates containing 1,400 μg/mL of ampicillin. Both mutants showed increased fluorescent responses in comparison to mutant 1A (Fig. 5A) when tested with the semi-quantitative fluorescence method. Cells expressing mutant 2A or 2B showed increased fluorescent responses over cells expressing mutant 1A at low cell densities, and the fluorescent responses of cells expressing 2A and 2B were comparable. At ODs over 0.4, all mutants exhibited similar fluorescence levels, indicating the saturation of GFP expression. However, the wildtype cultures exhibited lower fluoresecence intensities until OD 0.8 was reached. The difference between the second generation mutants (2A and 2B) and the first generation mutant (1A) were particularly apparent at low cell densities ($OD_{495} < 0.15$) where cells expressing mutant 2A and 2B exhibited significant fluorescence, while cells expressing mutant 1A or wildtype remained at basal levels of fluorescence. HPLC–MS/MS analysis indicated that the overall yields of mutant 2A and 2B were 25 times more than that of mutant 1A, or around 80 times more than that of the wildtype. Sequencing of the LuxI mutants showed that mutant 2A contains all of the mutations present in mutant 1A and 2B (Table I). A third round of mutagenesis with mutant 2B did not result in any further improvement of the LuxI enzyme.

Increased Production of C6HSL and C8HSL

It has been shown that LuxI synthesizes C6HSL and C8HSL in addition to the primary product OHHL (Kuo et al., 1994; Schaefer et al., 1996b). To determine if our mutants altered the specificity of the LuxI enzyme, we quantified the relative yields of C6HSL and C8HSL produced by the wildtype and mutant LuxI enzymes using HPLC–MS/MS. In the wildtype cultures there was not a detectable amount of C6HSL or C8HSL, but C6HSL was observed at significant concentrations in all the mutant cultures and C8HSL in the mutant 2A and 2B cultures (Fig. 5B). Compared to mutant 1A, both mutant 2A and 2B had increases in C6HSL production greater than 40-fold. Compared to the more than 25-fold increase in OHHL production, this result indicates that there was a slight change in substrate specificity as a result of the mutations.

Increased Expression Level of LuxI Mutants

To investigate whether the increase in OHHL production was caused by enhanced expression of LuxI, LuxI concentrations were determined by Western immunoblot assay. The wildtype and mutant LuxI genes were fused with a *c-myc* tag, and production of OHHL by the tagged LuxI variants confirmed their activity (data not shown). All mutants exhibit increased expression levels compared to the wildtype LuxI (Fig. 5C), and the expression levels of mutant 2A and 2B are higher than that of mutant 1A. Both mutant 2A and 2B show an approximate twofold increase in the expression level than the wildtype LuxI. Although the increase in the expression level is significant, it seems enhanced enzymatic

Table I. Nucleotide and amino acid substitutions of the LuxI variants and mutant relative activities.

LuxI variants	Nucleotide base substitution	Amino acid substitution	Relative OHHL yields
WT	N/A	N/A	1.0 ± 0.3
1A	A101G	E34G	3.1 ± 0.1
2A	A101G, A117T, A118G	E34G, V39V, E63G	78 ± 0.2
2B	A56G, A101G, A117T, A118G	K19R, E34G, V39V, E63G	84 ± 0.2

activity primarily contributes to the observed 80-fold increase in OHHL production by the LuxI mutants.

Discussion

As the first quorum-sensing system discovered in bacteria, the LuxI–LuxR system has been well characterized and used to construct a variety of artificial genetic circuits (Basu et al., 2005; Hooshangi et al., 2005; Kobayashi et al., 2004; You et al., 2004). The dynamics of these circuits are highly dependent on the kinetics of the LuxI–LuxR system, which in turn are determined by the kinetic properties of LuxI, LuxR, and the P_{luxI} promoter. Fine-tuning of the dynamics of a genetic circuit to achieve desired outputs without changing its architecture requires that the kinetics of its components are modified (Atkinson et al., 2003; Sayut et al., 2006; Yokobayashi et al., 2002). As such, the integration of directed evolution with synthetic biology appears to be an effective engineering strategy for the construction of functional genetic circuits (Sayut et al., 2007a; Yokobayashi et al., 2003). Alternatively, if kinetic variants of a genetic part are available, combinatorial assembly can be used to construct functional genetic circuits with desired outputs (Atsumi and Little, 2006; Guet et al., 2002). Engineering of LuxR for enhanced sensitivity to OHHL concentrations has been reported, and LuxR mutants capable of responding to a few molecules of OHHL per cell have been demonstrated (Collins et al., 2005; Sayut et al., 2006). Because of this high sensitivity, further enhancement of LuxR sensitivity is difficult. Therefore, to further improve the sensitivity of a cell culture with the LuxI–LuxR quorum-sensing system to cell population density, LuxI can be engineered to produce OHHL more efficiently so that the critical threshold concentration of OHHL is reached and the LuxI–LuxR system is activated at a much lower cell density than the wildtype system.

To use directed evolution for the engineering of LuxI variants with enhanced OHHL production, we developed a high throughput genetic selection. Selection was accomplished by using the P_{luxI} promoter to regulate a β-lactamase gene, and growing cells at increased concentrations of ampicillin. At elevated ampicillin concentrations, survival was dependent on the ability of the LuxI mutants to synthesize increased levels of the OHHL signal molecule and increase expression from the P_{luxI} promoter in comparison to the wildtype. In the absence of LuxR and OHHL, the P_{luxI} promoter exhibits a low basal expression level, as demonstrated previously (Sayut et al., 2006). In the presence of OHHL, a basal level of LuxR can activate the P_{luxI} promoter, a property consistent with its biological function in *V. fischeri* (Sayut et al., 2006). In our genetic selection method, both LuxR and LuxI genes are regulated by a $P_{ara/lac}$ promoter, and the basal expression of LuxR and LuxI in the absence of IPTG seems to be able to activate the P_{luxI} promoter, leading to cells growing in ampicillin concentrations (300 μg/mL) significantly higher than that commonly used in the selection of *E. coli* cells expressing β-lactamase (50–100 μg/mL) (Fig. 3A). Unfortunately, this genetic selection failed to identify more active mutants after two rounds of directed evolution. One possible reason could be that the P_{luxI} promoter is not strong enough to produce sufficient β-lactamase to rescue the cell at high concentrations of ampicillin (over 3,000 μg/mL). However, in the presence of 100 nM of OHHL, cells expressing the wildtype LuxI could grow on selection plates with 3,000 μg/mL of ampicillin (data not shown), indicating that the intrinsic activity of the P_{luxI} promoter is not the primary reason for not being able to identify more active LuxI mutants in the third round of directed evolution.

The in vivo semi-quantitative method is convenient for determining the activity of mutant LuxI enzymes relative to the wildtype. In cell cultures expressing the wildtype quorum-sensing system, fluorescence intensities remained at a basal level until the cell density reached an approximate OD_{595} of 0.3 after 5.5 h of growth. Once the quorum-sensing response was activated, fluorescence increased linearly with cell densities over a broad range (Fig. 5A). The observed linear fluorescent responses could be due to rapid activation of the P_{luxI} promoter by constitutively produced OHHL and LuxR which results in the saturated production of GFPuv. As a consequence, expression of GFPuv regulated by the P_{luxI} promoter is not enhanced despite OHHL is accumulated during cell growth. To verify if the saturated OHHL production rate was reached, 200 nM OHHL was spiked into a wildtype LuxI culture, and no significant change in fluorescence was observed (data not shown). Cell cultures containing LuxI mutant 1A exhibited significant fluorescent responses at an OD_{595} of 0.15 after 5 h of growth, followed by a linear increase with cell density until an OD_{595} of 1.2 was reached. Compared to the wildtype, which is activated at the cell density around 0.3, the mutant LuxI shows a significant increase in OHHL synthesis. This was further confirmed by HPLC–MS/MS analysis of the overall yields of cell cultures containing wildtype LuxI and mutant 1A, revealing a more than threefold increase in OHHL concentrations (Fig. 5B and Table I). Increased OHHL synthesis by mutant 2A and 2B was evident as the quorum-sensing systems were activated at cell optical densities of less than 0.1 after 3.5 h of growth in cell cultures expressing either mutant. Similar to the wildtype and mutant 1A, linear increases with cell density were observed for the fluorescent responses. The marked decrease in cell density required to activate the quorum-sensing response corresponds to a significant increase in the rate of OHHL synthesis, which resulted in more than a 25-fold increase in total OHHL levels for mutant 2A and 2B over mutant 1A as determined by HPLC–MS/MS. Because the quorum-sensing response is activated at such a low cell density, the use of the in vivo semi-quantification method in determining LuxI mutants more active than mutant 2A or 2B becomes unreliable. A change of culture conditions so that higher cell densities are required to activate the quorum-sensing system might be necessary to use this method for quantifying very active LuxI mutants.

Sequencing of the LuxI mutants revealed the amino acid substitutions present in the mutants that are responsible for the enhanced OHHL productions. A single amino acid substitution was introduced in mutant 1A and mutant 2A. Three amino acid substitutions occurred in mutant 2B including all the mutations in mutant 1A and mutant 2A (Table I). Mutant 2B and 2A also contained a synonymous mutation. Of the mutations, one (K19R) is located at the N-terminus, a region which has been proposed to interact with the SAM (*s*-adenosyl methionine) substrate (Watson et al., 2002). In addition, the mutation occurs in a highly conserved region of the AHL (acyl-homoserine lactone) synthase family (Parsek et al., 1997) that had been previously investigated using random mutagenesis to identify residues important for OHHL production (Hanzelka et al., 1997). Because there is not a significant difference in OHHL production between mutant 2A and 2B, the function of this mutation for increasing OHHL production seems minimal. Another mutation (D34G) is adjacent to a well-conserved amino acid residue (W35) involved in the formation of the substrate accessible tunnel (Gould et al., 2004; Pappas et al., 2004). In addition, the residue is conserved in LasI and EsaI (Gould et al., 2004; Watson et al., 2002). Consequently, this mutation most likely enhances the interactions between the enzyme and the acyl ACP substrate. Finally, mutation D63G is located in a β-sheet far from the amino acid residues essential for LuxI function, suggesting this mutation might enhance the folding of the enzyme to facilitate interactions between the substrates and enzyme. Previous site-directed mutagenesis of the *luxI* gene based on sequence analysis of the AHL synthase family did not generate any of the identified mutations (Hanzelka et al., 1997), though such a rational method has been used to alter the substrate specificity of a LuxI homolog (Brader et al., 2005). Despite not knowing the direct cause of the improvements in OHHL production, the mutants are expected to be useful for constructing engineered LuxI–LuxR quorum-sensing systems where enhanced OHHL production is desired (Kambam et al., 2007).

It has been demonstrated that three types of N-acyl homoserine lactones (OHHL, C6HSL, and C8HSL) are produced by LuxI (Kuo et al., 1994). OHHL is the cognate signaling molecule of LuxR as shown by the strong transcriptional activity induced by LuxR in the presence of OHHL (Schaefer et al., 1996a). Contrary to previous reports that similar levels of OHHL and C6HSL are present in *E. coli* cultures expressing LuxI (Schaefer et al., 1996b), our result indicates that OHHL is the dominant product of LuxI, at least in *E. coli* (Fig. 5B). In fact, C6HSL and C8HSL were not recovered from the wildtype LuxI culture presumably due to the low product yields and the low efficiency of the extraction method. However, significant amounts of both AHLs were detected in the mutant cultures, indicating increased production of all three AHLs by LuxI mutants. Slight change in substrate specificity due to the mutations remains unknown, and further research is required to illuminate the molecular basis.

In this report, we have described the development of a high throughput genetic selection and an in vivo semi-quantitative assay for the engineering of LuxI towards enhanced total activity by directed evolution. Using the genetic selection, we obtained a LuxI mutant with more than an 80-fold improvement in total activity. The improved activity was verified using the in vivo semi-quantitative assay, and further confirmed by HPLC–MS/MS analysis of the total OHHL yields in the cell cultures. Using Western immunoblot analysis, small but significant increases in the expression level of the LuxI mutants were observed. These methods do not distinguish between altered enzyme specific activity and modified expression levels, but are useful to determine the overall relative yields of mutant enzymes. As a consequence, the methods developed here are expected to have broad applications in engineering LuxI and its homologs. By replacing LuxI and LuxR with their corresponding homologs, the genetic selection can be used for the directed evolution of LuxI homologs for enhanced synthesis of AHLs. In addition, this high throughput method can also be used to alter the substrate specificity of LuxI homologs by using LuxR homologs that respond to the desired AHLs. The in vivo semi-quantitative assay of LuxI activity is simple and efficient compared to the time-consuming and expensive HPLC–MS/MS analysis. Together, the genetic selection and in vivo semi-quantitative analysis method will greatly facilitate future applications of directed evolution in engineering LuxI and its homologs.

This project is supported by an NSF CAREER Award (CBET 0747728) and a startup fund for L. Sun provided by the University of Massachusetts Amherst.

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