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A LuxP-FRET-Based Reporter for the Detection and Quantification of AI-2 Bacterial Quorum-Sensing Signal Compounds†

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ABSTRACT: Various bacterial species produce and monitor low-molecular weight signaling molecules that regulate specific sets of genes in a population density-dependent manner. This process is known as quorum sensing (QS). To date, the detection of QS signaling molecules from Gram-negative bacteria has relied primarily on bacterial reporter strains. These bioassays are subject to substantial interference by compounds that affect the growth and metabolism of the reporter strains. In addition, the sensitivity of reporter strains to QS signaling molecules is population density-dependent. Here, we describe the development of an in vitro assay system for the rapid detection and quantification of the furanosyl borate diester (BAI-2) subclass of autoinducer 2 (AI-2), QS molecules. The sensor is based on ligand binding-induced changes in fluorescence resonance energy transfer (FRET) between a cyan and yellow variant of GFP fused to the termini of the BAI-2 receptor, LuxP. Unexpectedly, the addition of synthetic BAI-2 to the purified biosensor induces a decrease in the level of FRET between the terminal fluorophores. Several lines of evidence, including mutation of the ligand binding sites, indicate that the observed FRET changes are BAI-2-dependent. The FRET-based BAI-2 biosensor responded to the addition of culture filtrates from wild-type *Vibrio harveyi* but exhibited no response to culture filtrates from *V. harveyi* mutants defective in BAI-2 synthesis. The sensitivity of the biosensor to BAI-2 (apparent K_d = 270 nM) was similar to that of BAI-2 bioassay systems. The limitations of microbial bioassay systems and the advantages and potential applications for the FRET-based BAI-2 biosensor are discussed.

Many bacterial species produce and monitor low-molecular weight signaling molecules to coordinate gene expression in a population density-dependent manner. This cooperative behavior is known as bacterial quorum sensing (QS),[1] and it controls the expression of a variety of genes. QS signal molecules may regulate a diverse array of functions, including antibiotic production, virulence, biofilm formation, stress and defense responses, motility, metabolism, and activities involved in interactions with eukaryotic hosts (*1*, *2*). Many bacterial species, including several *Vibrio* species, use multiple QS signaling pathways (*2*−*5*). *Vibrio harveyi*, an opportunistic pathogen of shrimp, is reported to use autoinducer 1 (AI-1), corresponding to *N*-(3-hydroxybutanoyl)-L-homoserine lactone, for intraspecies signaling and autoinducer 2 (AI-2), (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (BAI-2), for interspecies signaling (*6*). *V. harveyi* QS signals regulate gene expression systems involved in bioluminescence, type III secretion, siderophore production, colony morphology, and metalloprotease production (*7*−*10*).

The AI-2 class of QS signals is derived from 4,5-dihydroxy-2,3-pentanedione (DPD), which is enzymatically synthesized from *S*-ribosylhomocysteine. BAI-2 is formed from DPD by spontaneous cyclization with borate (*11*, *12*). In *V. harveyi*, BAI-2 binds to a periplasmic receptor protein, LuxP, which belongs to a large family of bacterial periplasmic binding proteins (bPBP) (*13*, *14*). Bacteria utilize a variety of bPBPs as signal receptors, chemosensors, and metabolite scavengers (*13*). The LuxP class of bPBPs is highly conserved among various *Vibrio* species which includes several potential human pathogens (Supporting Information). The crystal structure of the LuxP protein cocrystallized with its ligand BAI-2 has been determined. Interestingly, BAI-2 had not been identified prior to its structural resolution in the LuxP crystal structure (*12*). Recently, structures of the ligand-free form of LuxP, complexed with the periplasmic domain of its signaling partner, LuxQ, have also been determined (*6*).

Structure−function analyses of bPBPs in the presence and absence of their respective ligands have demonstrated that the binding of the ligand to its receptor induces substantial conformational changes in the receptor protein (*15*−*17*). Ligand binding can substantially reduce the protein radius of gyration and the distance between the N- and C-termini

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[1] Abbreviations: QS, quorum sensing; BAI-2, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate; DPD, 4,5-dihydroxy-2,3-pentanedione; AI-2, autoinducer 2; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; CLPY, CFP−LuxP−YFP fusion.

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of the protein (*13*−*17*). These ligand-induced structural changes have been exploited for the determination of apparent ligand binding affinities (K_d). Ligand-induced conformational changes in bPBPs have been monitored by spectral shifts in environmentally sensitive fluorescent dyes (e.g., acrylodan and fluorescein) that are covalently attached to the receptor protein (*13*). Similarly, biosensors that have blue or yellow versions of the jellyfish green fluorescent protein (GFP) translationally fused to the N- and C-termini of the receptor protein have been shown to undergo changes in fluorescence resonance energy transfer (FRET) between the fluorophores upon ligand binding. The resulting FRET-dependent changes in fluorescence emission ratios have been used to calculate apparent K_d values (*18, 19*).

Currently, bioassays for the detection of BAI-2 take several hours to complete and are subject to substantial environmental and biological perturbations. A more rapid in vitro assay for BAI-2 would be an attractive alternative. Here, we report the development of a highly sensitive, FRET-based BAI-2 biosensor. We have used this biosensor to quantify BAI-2 levels in *V. harveyi* cell culture filtrates and demonstrate that BAI-2 levels increase rapidly as the cell density increases followed by a decrease in BAI-2 levels before they reach a stationary phase. The BAI-2 biosensor has potential applications in monitoring bacterial population densities, including potential human pathogens.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media. *Escherichia coli* BL21 (*luxS*$^-$) and *V. harveyi* strains BB120 (wild-type), MM30 (*luxS*$^-$), and MM32 (*luxS*$^-$, *luxN*$^-$) used in this study were generously provided by B. L. Bassler (Princeton University, Princeton, NJ). Luria-Bertani (LB) medium was used to grow *E. coli*. Luria-Marine (LM) medium (*20*), autoinducer bioassay (AB) medium (*8*), and boron-free AB medium (*11*) were also used for the growth of *V. harveyi* strains. As needed, antibiotics ampicillin (100 mg/L) and kanamycin (100 mg/L) were used with growth media. For solid media, 15 g/L select agar (Invitrogen) was used with the liquid media. *V. harveyi* cell numbers were counted by plating serial dilutions of cultures on LM medium.

Site-Directed Mutagenesis and Cloning. Site-directed mutations were carried out using the QuikChange mutagenesis kit (Stratagene). The pcrGFP plasmid containing the gene encoding the green fluorescent protein (GFP) (*21*) was used for creating cyan and yellow variants CFP and YFP, respectively (*22*). An L221K mutation was introduced into the gene encoding CFP or YFP to reduce the possible level of intermolecular dimerization (*23*). Cloning vector pSP72 (Promega) was used for creating 5′ to 3′ end-to-end gene fusions of PCR fragments containing the genes encoding CFP (5′ *Kpn*I/3′ *Eag*I), *V. harveyi* LuxP (70−1095 bp; 5′ *Eag*I/3′ *Not*I), and YFP (5′ *Not*I/3′ *Pst*I), generating plasmid pSP72-CLPY. The CLPY gene fusion was removed using the restriction enzymes *Kpn*I and *Pst*I and subcloned into a pQE-30 bacterial expression vector (Qiagen) containing an N-terminal six-His tag, generating plasmid pQE30-CLPY. LuxP BAI-2 binding site mutants pQE30-M2CLPY (Q77A and S79A) and pQE30-M3CLPY (Q77A, S79A, and W82F) were created using pQE30-CLPY as a template and confirmed by DNA sequence analysis.

Protein Overexpression, Purification, and Characterization. BL21 (*luxS*$^-$) cells transformed with pQE30-CLPY or LuxP mutant constructs were grown at 28 °C in LB broth supplemented with 100 mg/L ampicillin. Protein expression was induced with 0.3 mM isopropyl thiogalactoside at an optical density (OD_{600}) of 0.6 and grown for an additional 6 h. Bacterial cells were harvested by centrifugation, and further purifications were carried out at 4 °C. Cells were resuspended in 25 mM NaH_2PO_4-Na_2HPO_4 (pH 8.0), 35 mM NaCl, and 10 mM imidazole (buffer A) and sonicated with 15 mM 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride. Clarified cell lysate was then loaded onto a His-Select-HC nickel affinity gel (Sigma) equilibrated with buffer A. The protein-bound resin was washed with buffer A and eluted with 25 mM NaH_2PO_4-Na_2HPO_4 (pH 8.0), 35 mM NaCl, and 50 mM imidazole (buffer B). The protein purity was judged by SDS−PAGE, and the protein yield was determined using a Bradford protein assay kit (Bio-Rad). Fluorescence measurements were carried out at room temperature using a Cary Eclipse spectrofluorometer (Varian Inc.) set in a scanning mode. Wild-type and mutant forms of CLPY were monitored on the column by CFP fluorescence emission (λ_{ex} = 440 nm, 5 nm slit; λ_{em} = 460−560 nm, 5 nm slit). Separation of CLPY monomers and dimers was carried out by size-exclusion chromatography using a FPLC system fitted with a Sephacryl S-100 HR 26/60 column (Amersham Biosciences). The column was equilibrated with buffer B, and 500 μL of 2.5 mg/mL CLPY protein was applied at a flow rate of 0.8 mL/min. Protein standards [thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa)] (Bio-Rad) were run under similar conditions.

Synthesis of a Boron Derivative of Autoinducer 2 (BAI-2). Nucleosidase Pfs and Co^{2+}-substituted *S*-ribosylhomocysteinase (LuxS), both from *Bacillus subtilis*, were overexpressed in *E. coli* BL21(DE3) and purified to apparent homogeneity as previously described (*24*). *S*-Adenosylhomocysteine (SAH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). SAH (15 mM) was incubated with nucleosidase Pfs (4 μM) for 6 h at room temperature to give *S*-ribosylhomocysteine (SRH), and the completion of the reaction was monitored spectrophotometrically on the basis of the absorption difference between SAH and adenine ($\Delta\epsilon_{276} = -1.4$ mM^{-1} cm^{-1}) (*25*). The crude SRH was directly used in LuxS reactions without further treatment. BAI-2 was prepared by incubating SRH (3 mM) and LuxS (2.0−2.5 mg/mL) at room temperature overnight in 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.5), 20 mg/mL $NaHCO_3$, and 12 mM borate (4 equiv). The DPD yield was determined indirectly by assaying the amount of homocysteine released using DTNB (*26*), and the concentration of BAI-2 was corrected by a factor of 0.1 on the basis of the ^{11}B NMR analyses (*27*). DPD was synthesized as described above without the addition of borate in the LuxS reaction buffer.

Binding Affinity Determination. The YFP or CFP fluorescence response of wild-type and mutant forms of CLPY (1.0 mL, 0.015 mg/mL) to different ligands was measured 5 min after addition of the ligand to the protein at room temperature. No time-dependent changes in the CLPY FRET ratio were observed after incubation for 5 min. All BAI-2

dilutions were carried out in a borate-free buffer to maintain the DPD:borate ratio (1:4). The CLPY FRET ratio response to different BAI-2 concentrations was used to generate the ligand binding saturation curve. For the determination of the binding affinity (K_d) and the binding stoichiometry n, a nonlinear regression analysis equation (eq 1) was used as reported elsewhere (*18*, *19*).

$$R = R_{max} - [(R_{max} - R_{min})ns]/(K_d + s) \quad (1)$$

where R is the observed CLPY FRET ratio response for different ligand concentrations (s) and R_{max} and R_{min} represent the ligand-free and ligand-saturated FRET ratios, respectively.

BAI-2 Ligand Detection and Quantification from V. harveyi Cultures. For the detection of BAI-2 in *V. harveyi* cultures, BB120 (wild-type) and MM30 (*luxS*$^-$) cells were used. *V. harveyi* was grown overnight at 28 °C for 16 h and used to make a 2% (v/v) inoculum in fresh AB medium (2.0 mL, 0.5 mM borate) in round-bottom polystyrene tubes (BD Biosciences). The cell density was determined by plating serial dilutions of *V. harveyi* cultures on LM agar plates. Every 2.5 h, the BB120 cell-free supernatant was collected by centrifugation at 4 °C for BAI-2 detection. To remove proteins (proteases) and cell debris, the supernatant was passed through a 3 kDa membrane cutoff filter (3K Microsep centrifugal device, Pall Life Science). For the determination of the unknown BAI-2 concentration, 200 μL of various dilutions of a 7.5 h old *V. harveyi* BB120 (wild-type) culture supernatant grown in borate-free AB medium was added to CLPY (1.0 mL, 0.015 mg/mL) to generate a saturation curve. Cell-free supernatant from a 7.5 h old *V. harveyi* MM32 culture was used as a control. Using a nonlinear regression relationship similar to eq 1 (see eq 2), the CLPY response to various dilutions of *V. harveyi* supernatant v was used in determining the half-saturation volume h.

$$R = R_{max} - [(R_{max} - R_{min})nv]/(h + v) \quad (2)$$

The unknown BAI-2 concentration M was determined using the relationship

$$M = K_d/h \quad (3)$$

Bacterial Reporter Bioassay and EC_{50} Determination. V. harveyi strain MM30 (*luxS*$^-$) was grown overnight at 28 °C for 16 h and diluted 1:5000 in fresh borate-free AB medium. Bacteria (90 μL) were added to a 96-well microtiter plate containing 10 μL dilutions of the sample and incubated for 4 h at 30 °C (*28*). Luminescence was read using a Wallac Victor2 multimode plate reader (Perkin-Elmer). The BAI-2 concentration required for 50% maximum luminescence response (EC_{50}) was determined using a relationship based on the luminescence response L for different ligand concentrations s

$$L = L_{min} + [(L_{max} - L_{min})s]/(EC_{50} + s) \quad (4)$$

where L_{min} and L_{max} represent the minimum and maximum luminescence responses, respectively, at the time of measurement.

RESULTS

Characterization of the FRET-Based BAI-2 Biosensor. The bPBPs typically have two globular protein domains tethered by a flexible hinge region that encompasses the ligand binding site (*14*). The two globular domains move about the hinge region in response to ligand binding. To monitor the binding of BAI-2 to LuxP, we fused a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) to the surface-exposed N- and C-termini of the LuxP protein (Figure 1A). In addition, a six-His tag was included at the N-terminus of the CLPY biosensor for rapid metal affinity purification. To ensure isolation of a ligand-free biosensor complex, the protein was expressed in an *E. coli* mutant (luxS$^-$) which is unable to synthesize BAI-2. We also introduced a mutation (L221K) into the CFP and YFP genes to reduce the potential level of fluorophore dimerization and associated FRET artifacts (*23*). It is noted that maturation of YFP fluorescence yield required incubation for an additional 5 h at 21−23 °C after its extraction and purification from *E. coli* due to its delayed chromophore maturation. The mature BAI-2 biosensor protein fusion is designated CLPY.

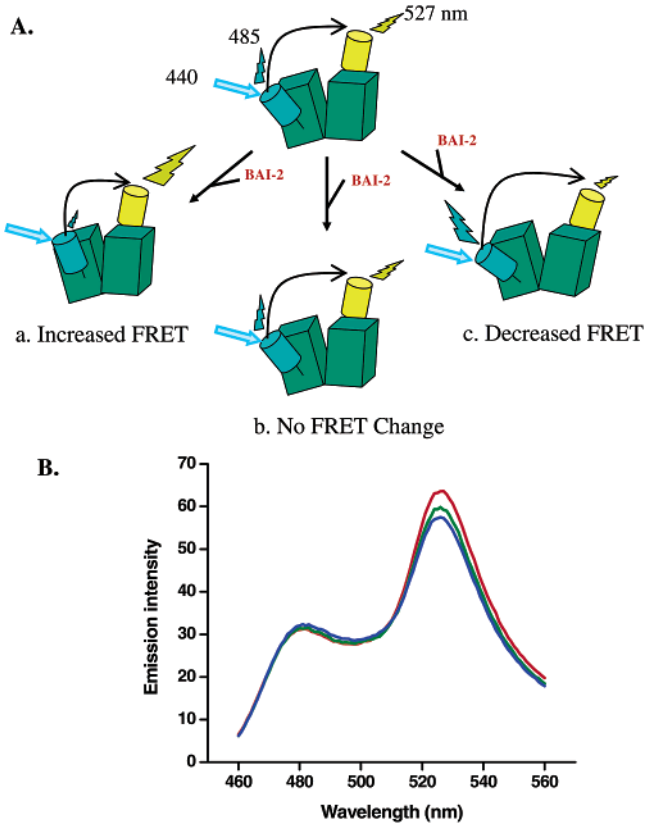

FIGURE 1: Possible mechanisms for ligand-induced FRET changes in CLPY. (A) Schematic representing the CLPY protein as a CFP (cyan), LuxP (green), YFP (yellow) fusion protein. In the absence of BAI-2, CFP exhibits high fluorescence. There are three possible outcomes following ligand binding (a−c) associated with either a conformational-induced change in the distance between CFP and YFP, a change in the orientation of the chromophores, or no change at all (b). (B) Response of CLPY to unsaturating [1.0 nM (red)], partially saturating [285 nM (green)], and fully saturating [5.6 μM (blue)] concentrations of BAI-2 ligand.

Addition of BAI-2 to CLPY resulted in a nonlinear decrease in the YFP:CFP fluorescence ratio up to the point of substrate saturation (Figure 1A,B). Previous studies have shown that binding of ligand to other bPBP proteins can increase or decrease the distance between the N- and C-termini, resulting in a decreased or increased FRET

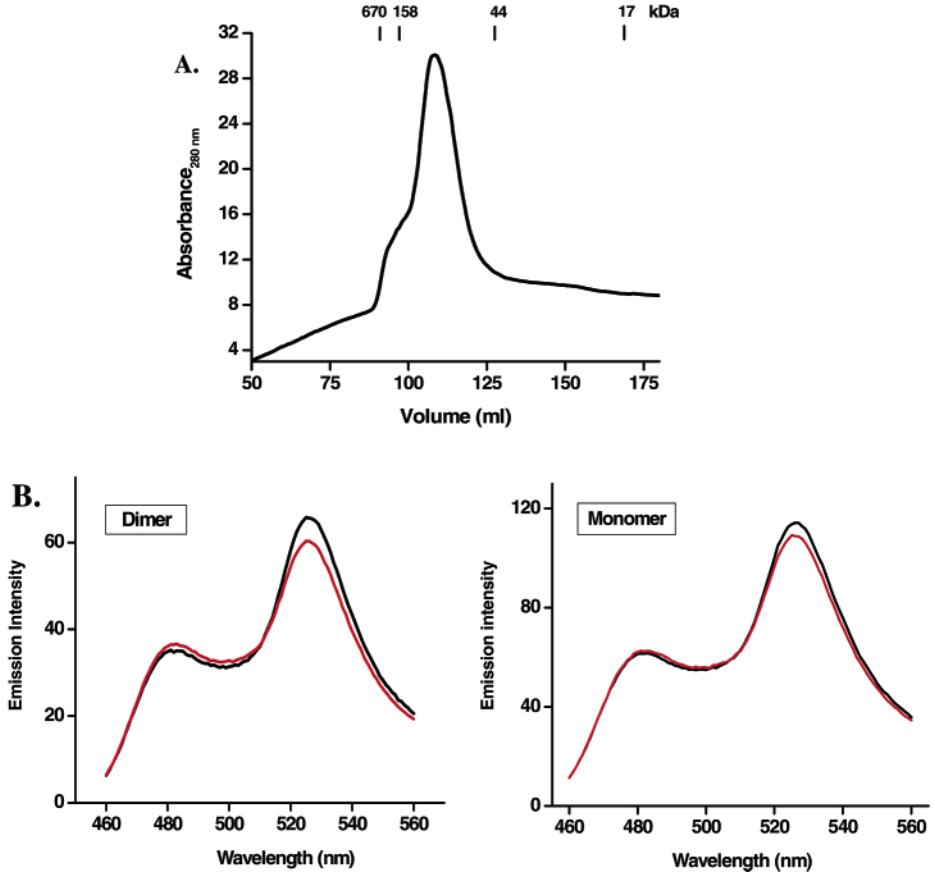

FIGURE 2: CLPY functions as a monomeric biosensor. (A) Size-exclusion chromatography elution profile of CLPY protein (1.25 mg of protein applied). The protein was detected at 280 nm and was found to exist predominantly in the monomeric state (98 kDa). (B) FRET responses of dimeric (91−99 mL) and monomeric (106−114 mL) CLPY to buffer (black) and 1.5 μM BAI-2 (red). The observed differences in the magnitude of the response between the dimeric and monomeric fractions (for added 1.5 μM BAI-2) are attributed to dissimilar protein concentrations in the representative samples.

response, respectively (*18*, *19*). On the basis of the crystal structure model of ligand-complexed LuxP, the CFP and YFP domains of the BAI-2 biosensor are predicted to be sufficiently close (37 Å) to facilitate efficient FRET. Therefore, a decrease in the FRET ratio following ligand binding implies that the fluorophores either move away from each other or substantially change their optical orientations. Analyses of the distances between the N- and C-termini of LuxP determined from the crystal structures of holoLuxP, with bound BAI-2, and apoLuxP, complexed with the periplasmic domain of LuxQ, indicate that there are no appreciable changes in the distance between the N- and C-termini of LuxP with or without bound BAI-2 (*6*, *12*). Since FRET efficiency differs with respect to distance (r) by $1/r^6$, however, even small distance changes between the N- and C-termini may have large effects on FRET ratios.

Changes in FRET ratios upon BAI-2 binding could also be attributed to intermolecular FRET due to end-to-end dimerization of CLPY. Furthermore, binding of ligand to LuxP has been shown to induce dissociation of LuxP dimers, potentially leading to a reduced level of intermolecular FRET (*6*). Therefore, a decrease in the level of FRET upon BAI-2 binding could be attributed to either a ligand binding-induced conformational change in LuxP (Figure 1, mechanism c) or a shift from a dimeric to monomeric state. To determine if the loss of FRET was associated with a shift from a dimeric to monomeric state, we compared the YFP:CFP fluorescence ratios of monomeric and dimeric CLPY separated by size-exclusion chromatography. Isolated CLPY was determined to be predominantly in a monomeric state (Figure 2A). Importantly, ligand-induced changes in the YFP:CFP fluorescence ratios were identical for monomeric (volumes of 106−114 mL) and dimeric (volumes of 91−99 mL) forms of CLPY (Figure 2B). These results suggest that intermolecular dimerization of CLPY does not contribute to the observed ligand binding-induced changes in FRET.

CLPY Binding Affinity for BAI-2. To determine the affinity of the CLPY biosensor for BAI-2, the protein was incubated with various concentrations of BAI-2 and the FRET response was measured. The maximum ligand-induced change in the YFP:CFP fluorescence emission ratio was ~0.32 (Figure 3A). Nonlinear regression analysis of the concentration-dependent FRET response demonstrated that the apparent K_d for BAI-2 was 270 nM with a binding stoichiometry of 1.2 BAI-2 per molecule of CLPY. This high binding affinity is consistent with the ligand affinity constants measured for other bPBPs (*13*, *18*, *19*). To determine if the CLPY FRET response was specifically due to BAI-2 binding and not to interactions with structurally similar signaling molecules such as the AI-2 synthesis intermediate, DPD, FRET responses were measured in the presence of BAI-2 synthesis intermediates. CLPY did not exhibit any detectable FRET response to DPD ($\leq$100 μM) or borate ($\leq$400 μM) at high concentrations (Figure 3A). To demonstrate that the

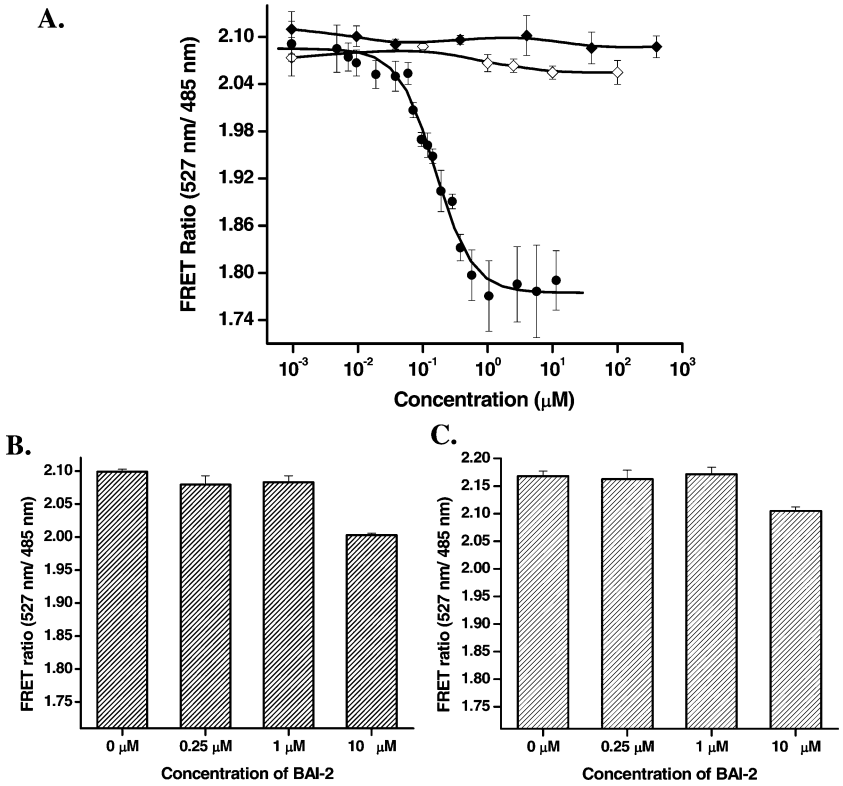

FIGURE 3: CLPY responds specifically to BAI-2. (A) FRET response of CLPY to different concentrations of BAI-2 in borate-free buffer (●), DPD (◇), and borate (◆). The error bars represent the standard deviations calculated from the FRET assays from three independent experiments. (B) Response of LuxP M2CLPY to different concentrations of BAI-2. (C) Response of LuxP M3CLPY to different concentrations of BAI-2.

BAI-2-dependent FRET response was due specifically to binding of BAI-2 to its LuxP binding site, a series of ligand binding site mutants were created. Site-specific mutations were introduced into the LuxP protein at several ligand binding site positions, including Q77A and S79A (designated M2CLPY) and Q77A, S79A, and W82F (designated M3CLPY). In the ligand-free state, both mature mutant biosensor proteins exhibited YFP:CFP ratios slightly higher than that of the wild-type protein. These FRET ratio increases may be attributed to the replacement of the glutamine and tryptophan side chains with smaller side chains such as alanine and phenylalanine, respectively. Following addition of BAI-2, both CLPY mutants had substantially reduced FRET responses compared to the wild-type CLPY protein. BAI-2 concentrations (≥ 1 μM) that yielded a maximum FRET response with the wild-type biosensor had little effect on the YFP:CFP ratio of the CLPY mutants. At supersaturating concentrations of BAI-2 (10 μM), the mutant biosensors M2CLPY and M3CLPY had only 30 and 18%, respectively, of the FRET response of the wild-type biosensor (Figure 3B,C). These results indicate that the BAI-2-dependent reductions in the CLPY YFP:CFP ratio are not associated with binding of BAI-2 to nonspecific binding site(s).

Comparison of CLPY Biosensor and Bacterial Reporter Bioassay Responses to BAI-2. One potential advantage of bacterial bioassay systems is potential amplification of the signal response following addition of the elicitor. To determine if the CLPY biosensor was as sensitive as the bacterial bioassay system for BAI-2, we compared the BAI-2 concentration-dependent response (luminescence) of the *V. harveyi* reporter strain, MM30, to the BAI-2-induced FRET response of the CLPY biosensor. In addition, we compared the relative responses of the two assay systems to DPD and borate. To prevent DPD from spontaneously forming BAI-2 in the presence of boron, *V. harveyi* MM30 cells were grown in borate-free AB medium (*11*). As shown in Figure 4, the bacterial reporter strain exhibited a BAI-2 concentration-dependent response but did not respond to the addition of DPD or borate (Figure 4). Using a nonlinear regression model, the EC_{50} (50% effective luminescence yield concentration) bioluminescence response for BAI-2 was determined to be 100 nM. The *V. harveyi* bioluminescence response to

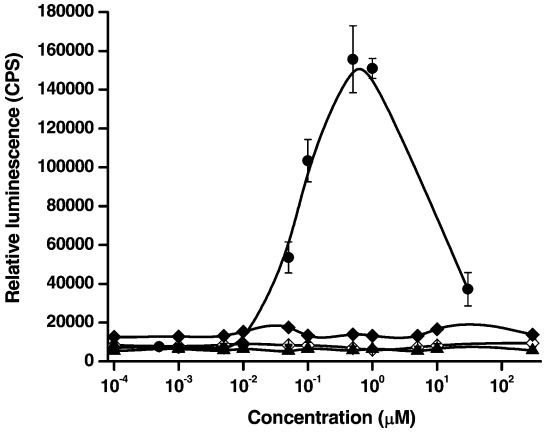

FIGURE 4: Response of *V. harveyi* MM30 (*luxS*$^-$) reporter strain bioluminescence to synthetic BAI-2 (●), DPD (◇), borate buffer (◆), and blank (▲). Each data point represents an average of three repeats for each sample concentration.

BAI-2 was ~2.7 fold lower than the K_d (270 nM) of CLPY for BAI-2 (Figure 3A). As observed in Figure 4, however, the bioluminescence yield decreased with BAI-2 concentrations of >0.5 μM. The reduction in bioluminescence at high BAI-2 concentrations clearly demonstrates one of the limitations associated with in vivo bacterial bioassay systems (*29*, *30*). Unlike the bacterial system, the CLPY biosensor exhibited no reduction in FRET response in the presence of supersaturating ($\leq$10 μM) concentrations of BAI-2 (Figure 3A). These results imply that the reduction in bioluminescence in bacterial strains exposed to high concentrations of BAI-2 is not directly mediated by LuxP but may involve a feedback inhibition pathway or expression of a repressor that reduces the bioluminescence response.

Quantification of BAI-2 in Bacterial Culture Filtrates. We then used the CLPY biosensor to determine BAI-2 levels in culture filtrates of wild-type *V. harveyi* (BB120) cells. Early to late log phase cultures exhibited a population-dependent increase in BAI-2 levels as indicated by a decrease in the YFP:CFP fluorescence intensity ratio (Figure 5A). The highest BAI-2 levels were observed at a culture density of ~1×10^9 cfu/mL (7.5 h) (Figure 5A). At this culture density, the experimentally determined BAI-2 concentration was 4.2 μM (Figure 5B). Culture filtrates obtained from LuxS$^-$ mutant (MM32) *V. harveyi* cultures that are unable to synthesize BAI-2 did not induce a change in the YFP:CFP ratio of the CLPY biosensor, indicating that the response was specific for BAI-2 and that other molecules in the growth media had no effect on the biosensor's BAI-2-dependent FRET response. Interestingly, as the culture aged, the BAI-2 levels decreased (10−20 h, Figure 5A). Our results confirm the age-dependent decreases in BAI-2-mediated bacterial reporter responses observed earlier with *V. harveyi* cultures (*31*). An analogous age-dependent AI-2 production decrease has also been observed with organisms such as *Vibrio vulnificus*, *Salmonella typhimurium*, and *E. coli* cultures (*31*−*33*). It is evident that *V. harveyi* actively synthesizes BAI-2 during the early logarithmic phase of culture growth, but at latter stages, BAI-2 levels drop, reflecting either an increased level of partitioning into the bacterial biomass or accelerated turnover.

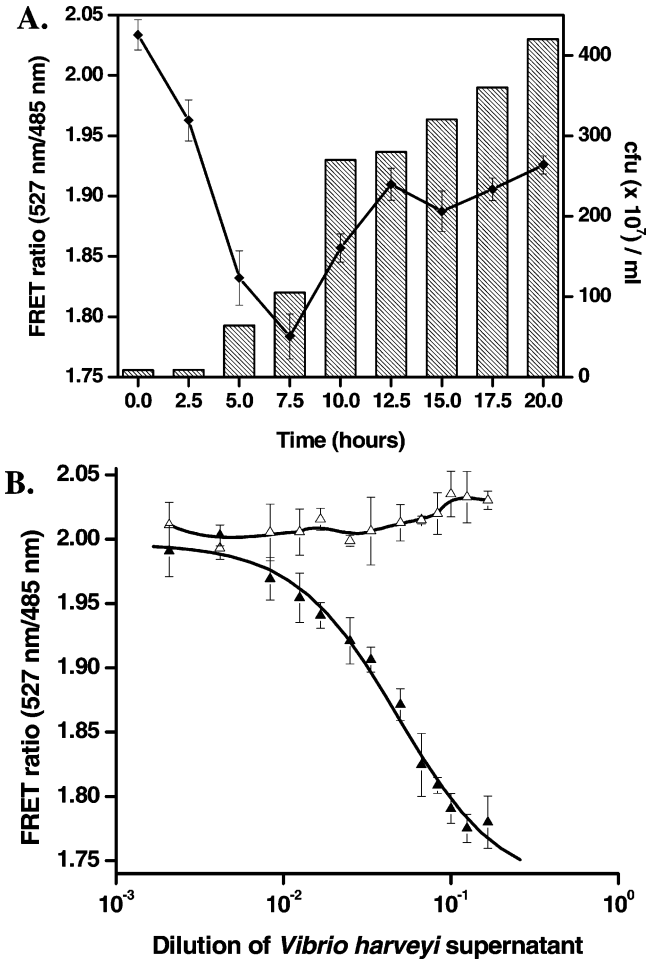

FIGURE 5: Quantification of BAI-2 from *V. harveyi* cultures using the CLPY biosensor. (A) Wild-type *V. harveyi* BAI-2 levels monitored as a function of time and culture density. Cell-free culture filtrates were prepared by passing the bacterial cell culture medium through a 3 kDa molecular mass cutoff filter. The filtrate (150 μL) was added to CLPY (0.015 mg/mL). The YFP:CFP FRET ratio (◆) is plotted as a function of cell density ($\times 10^7$ cfu/mL; bars). The error bars represent the standard deviations from three independent experiments. (B) Response of CLPY fluorescence to culture filtrate from wild-type (BB120) *V. harveyi* (▲) and *LuxS*$^-$ mutant (MM32, △) mid- to late-log phase culture filtrate. The error bars represent the standard deviations from three independent experiments.

DISCUSSION

Currently, bacterial reporter bioassays are typically employed for the detection and quantification of QS signaling molecules. Bacterial reporter strains have also been used to detect compounds that interfere with QS and/or QS mimics produced by higher organisms (*34*−*37*). QS bioassay systems are, however, subject to substantial environmental perturbations. Previously, we observed that the detection of AHL QS mimics produced by *Chlamydomonas reinhardtii* and detected using a *V. harveyi* reporter strain (BB170, *luxN*$^-$) exhibited large disparities in reporter response between replicate experiments (unpublished data). Similar observations have been made by other groups using bacterial bioassay systems (*29*, *30*). Fluctuations in culture pH and metabolite and growth inhibitor concentrations may all affect QS bioassays (*29*, *30*). These limitations clearly emphasize the need for caution when interpreting the results from different bioassay experiments.

In contrast to bacterial reporter assay systems, the CLPY biosensor undergoes a highly reproducible shift in the YFP:CFP fluorescence ratio upon ligand binding. This ligand-dependent reduction in the FRET ratio is associated with an intramolecular conformational change in the LuxP protein following ligand binding possibly involving a decrease in the distance between the N- and C-termini of LuxP (Figures 1B and 2). Control experiments with BAI-2 precursors and LuxP BAI-2 binding site mutations demonstrate that the biosensor response is specific for BAI-2 (Figure 3). For the first time, we have measured an apparent in vitro K_d for synthetic BAI-2 (270 nM) bound to LuxP. The affinity of LuxP for BAI-2 is consistent with expectations from bacterial reporter bioassay experiments.

Interestingly, analyses of BAI-2 concentrations in wild-type *V. harveyi* cultures indicate that BAI-2 levels initially increase as the culture grows to mid-log phase and then decrease as the culture ages and its density increases (Figure 5). These results suggest that rapid induction of QS-mediated gene expression may be critical for effective

regulation of QS-mediated responses. However, even at high culture densities, the effective BAI-2 concentration is sufficient to induce a QS response on the basis of the affinity of LuxP for BAI-2. Finally, the CLPY biosensor offers many advantages over current bioassays for the detection and quantification of BAI-2 QS compounds. The CLPY assay system is rapid (5 min) relative to conventional BAI-2 bioassays (3−6 h) (Figure 4), and it is not subject to a reduced signal response at high BAI-2 concentrations (Figure 3A). These attributes are particularly useful for monitoring BAI-2 levels in bacterial cultures as well as in fluids obtained from infected tissues. In this latter capacity, BAI-2 biosensors may be used to monitor indirectly and noninvasively infection states in host organisms and the efficacy of treatments used to control those infections. Currently, efforts to use the CLPY biosensor to screen for bacterial QS mimics and synthetic analogues of BAI-2 that bind to LuxP are underway. Along this line, LsrB, a paralog of LuxP found in *S. typhimurium* that binds specifically to the non-borote form of AI-2 (*11*), could potentially be utilized in the development of a second class of in vitro QS reporter systems. The LuxP and LsrB reporter pair will then provide useful information delineating specifically each class of AI-2 signaling molecule present at a given time of bacterial culture age and growth conditions.

SUPPORTING INFORMATION AVAILABLE

Multiple-sequence alignment of LuxP homologues from various *Vibrio* species. This material is available free of charge via the Internet at http://pubs.acs.org.

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