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A sensitive fluorescence reporter for monitoring quorum sensing regulated protease production in *Vibrio harveyi*

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ABSTRACT

Many bacteria produce and secrete proteases during host invasion and pathogenesis. *Vibrio harveyi,* an opportunistic pathogen of shrimp, is known to use a two-component quorum sensing (QS) mechanism for coordination of gene expression including protease secretion at high population densities. We examined the role of *V. harveyi's* QS signaling molecules, N-(3-hydroxybutanoyl)-L-homoserine lactone (AI-1) and the boron derivative of autoinducer-2 (BAI-2) in extracellular protease production. A fusion protein, M3CLPY (Rajamani et al., 2007), consisting of a large protease sensitive BAI-2 mutant receptor LuxP (~38 kDa) flanked by two protease insensitive cyan and yellow variants of GFP (~28 kDa each) was utilized as a substrate to detect secreted protease activity. The M3CLPY fusion, with the addition of wild-type *V. harveyi* (BB120) cell-free culture filtrate showed a time-dependent loss in fluorescence resonance energy transfer (FRET) associated with the cleavage of the LuxP linker protein and hence separation of the two flurophores. This cleavage of LuxP linker protein leading to decreased FRET efficiency was further confirmed by immunoblotting using anti-GFP antibody. The addition of cell-free filtrates from strains defective in one or both of the two-component QS pathways: *luxN*$^-$ (defective in AI-1), *luxS*$^-$ (defective in BAI-2), and *luxN*$^-$/*luxS*$^-$ (defective in both AI-1/BAI-2) showed differential levels of protease production. The observed protease activities were most pronounced in wild-type, followed by the AI-1 defective mutant (BB170) and the least for *luxS*$^-$ mutant (MM30) and *luxN*$^-$/*luxS*$^-$ double mutant (MM32) strains. Incidentally, the lowest protease producing strains MM30 and MM32 were both defective in BAI-2 production. This observation was validated by addition of synthetic BAI-2 to MM30 and MM32 strains to restore protease production. Our results indicate that BAI-2 signaling in the two-component QS pathway plays the key role in regulating extracellular protease production in *V. harveyi.*

1. Introduction

Bacterial quorum sensing (QS) involves the production, secretion, and detection of specific signal molecules to regulate gene expression. The concentration of QS signals increases as the number of bacteria in a local population increase. Upon reaching a threshold concentration, the signaling molecules activate specific receptors to regulate QS-dependent gene expression (Bassler and Losick, 2006; Miller and Bassler, 2001). Gram-negative bacterial species produce one or more QS signal molecules, most commonly N-acyl homoserine lactones (AHLs) and derivatives of 4,5-dihydroxy-2,3-pentanedione (Bassler and Losick, 2006; Henke and Bassler, 2004a,b; Miller and Bassler, 2001; Schauder et al., 2001).

The bioluminescent opportunistic shrimp pathogen, *Vibrio harveyi,* is a good model for studying dual signal QS regulation. *V. harveyi* utilizes an AHL, N-(3-hydroxybutanoyl)-L-homoserine lactone (AI-1) for intraspecies signaling and (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (BAI-2) for interspecies signaling (Mok et al., 2003; Neiditch et al., 2005). This dual two-component QS regulation has also been found conserved in several other marine and human pathogenic *Vibrios* including *V. cholera, V. vulnificus* and *V. parahaemolyticus* (Henke and Bassler, 2004a,b; Kim et al., 2003; Miller et al., 2002). AHL detection relies on a transmembrane sensor histidine kinase receptor LuxN and BAI-2 signal detection depends on a periplasmic receptor protein LuxP found complexed with LuxQ, a sensor histidine kinase (Miller and Bassler, 2001; Neiditch et al., 2005; Waters and Bassler, 2005; Whitehead et al., 2001).

The current model for *V. harveyi* quorum sensing involves a low population density-dependent initiation of the histidine kinase activities of LuxN and LuxQ. The phosphorylated sensor kinases

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initiate a phosphor-relay, converging on to a regulator protein LuxO. The phosphorylated LuxO, in a mechanism utilizing regulatory small RNAs and chaperones, down regulates expression of the transcription regulator, LuxR, a protein different from the LuxR receptor used for AHL detection in other bacteria. Upon reaching a high population density, AI-1 binding to the AHL receptor LuxN, or BAI-2 binding to receptor LuxP, induces dephosphorylation of the phosphorylated proteins, resulting in LuxR stabilization and gene expression (Waters and Bassler, 2005). Some of the genes that are regulated in response to this signaling mechanism include bioluminescence, inhibition of type III protein secretion, biofilm formation and increased production of a metalloprotease (Bassler et al., 1993; Henke and Bassler, 2004a,b; Lilley and Bassler, 2000; Mok et al., 2003). This *V. harveyi* metalloprotease was determined to be highly up-regulated only in the presence of both QS signals (Mok et al., 2003).

Protease production and secretion play a critical role in the host invasion processes, and their regulation is complex (Lory, 1998; Pugsley, 1993; Scott et al., 2001). Earlier studies have identified a cysteine protease and one or more metalloproteases in *V. harveyi* extracellular media (Fukasawa et al., 1988a,b; Fukasawa et al., 1988a, b; Lee et al., 1999; Liu et al., 1997). Given the importance of QS and virulence, we investigated the secretion of extracellular proteases by *V. harveyi* mediated by a QS mechanism. Further, in this study, we attempted to determine the roles for individual QS sensing signal molecules (AI-1 and BAI-2) in *V. harveyi* protease production using a fluorescence resonance energy transfer (FRET) based biosensor. FRET based reporters have been previously used to monitor and quantify protease activity. For instance, to monitor caspase activity, a short peptide with a known protease target sequence flanked by protease insensitive GFP variants was used as FRET indicator (Mahajan et al., 1999; Xu et al., 1998). To monitor the extracellular protease activity from *V. harveyi,* we employed a novel strategy that utilized LuxP, a bulky bacterial periplasmic binding protein (bPBP) as a general target for proteases. Our protease FRET reporter labeled M3CLPY consists of BAI-2 receptor mutant protein LuxP of *V. harveyi* (carrying point mutations that abolished BAI-2 binding; also refer to Rajamani et al., 2007) fused to N-terminal cyan fluorescent protein (CFP) and C-terminal yellow fluorescent protein (YFP). Using this novel FRET reporter assay we probed the individual and collective roles for QS molecules in the extracellular protease secretion in wild-type and QS mutant cultures of *V. harveyi*.

2. Materials and methods

2.1. Bacterial strains and media

Escherichia coli BL21 (*luxS*$^-$) and *Vibrio harveyi* strains BB120 (wild type), MM30 (*luxS*$^-$), BB170 (*luxN*$^-$), and MM32 (*luxS*$^-$, and *luxN*$^-$) were obtained from ATCC or generously provided by Dr. Bonnie L. Bassler (Princeton University). Luria–Bertani (LB) media was used to grow *E. coli* while Luria–Marine (LM) media (Bassler *et al.*, 1994), Autoinducer Bioassay (AB) media (Bassler et al., 1993), and boron-free AB media (Miller *et al.*, 2004) were used for *V. harveyi* stains. As needed, antibiotics ampicillin (100 mg/L) and kanamycin (100 mg/L) were used with growth media. For solid media, 15 g/L of select agar (Invitrogen) was used with the liquid media. *V. harveyi* cell numbers were counted by plating dilutions of cultures in LM agar plates.

2.2. Protein overexpression, purification and characterization

E. coli BL21 (*luxS*$^-$) transformed with the pQE30-M3CLPY (as detailed in (Rajamani et al., 2007)) construct was grown at 28 °C in LB broth supplemented with 100 mg/L ampicillin. Protein expression was induced with 0.3 mM isopropyl thiogalactoside (IPTG) at an OD_{600} of 0.6 and grown for an additional 6 hours. Bacterial cells were harvested by centrifugation and M3CLPY protein purification was 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 1.0 mM phenylmethyl sulfonyl 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). Protein purity was judged by SDS/PAGE and protein concentrations determined with Bradford protein assay kit (BioRad). Fluorescence measurements were carried out at room temperature using a Cary Eclipse spectroflurometer (Varian Inc) set in a scanning mode. M3CLPY was excited (λ_{ex} 440 nm, slit 5 nm) and emission spectra were collected (λ_{em} 460–560 nm, slit 5 nm).

2.3. Protease assay with cell-culture filtrate and autoinducer treatment

V. harveyi strains grown overnight at 28 °C for 16 hours were diluted 50× in fresh AB media (2%) and grown for additional 16 hours. The cell-free supernatant was collected by centrifugation at 4 °C. The supernatant was passed through a 0.2 μm HT Tuffryn® membrane syringe (Pall Life Science). For monitoring extracellular protease activity, 250 μL of cell-free filtrate mixed with 1.0 mL of 0.015 mg/mL M3CLPY and was used for the FRET assays. Synthesis of the boron derivative of DPD and its boron derivative BAI-2 were carried out as described previously (Rajamani et al., 2007). For the extracellular protease recovery study, the *V. harveyi luxS*$^-$ strains MM30 and MM32 were grown overnight in borate-free AB media for 16 hours at 28 °C. The overnight cultures were 50× diluted in fresh borate-free media and were grown for 4.5 hours before 10 μM BAI-2 or borate containing buffer was added and cultures were grown for additional 11.5 hours. The BAI-2 or buffer treated cell-free culture filtrate were prepared as before and tested for protease activity using the FRET assay.

2.4. Western blotting

Western blotting analysis was carried out using equal amounts (150 ng) of M3CLPY from protease assay experiments. A 10% SDS-PAGE was used to resolve the proteins and the resolved gel was transferred to Immobilin-P (PVDF) membrane (Millipore) by a semi-dry blot transfer. The membrane was washed with Tris-buffered saline (TBS) solution containing 0.05% Tween 20 and blocked with TBS containing 2% dry milk. The membrane was incubated with 1 to 1000 dilution of primary anti-GFP (goat) antibody conjugated with alkaline phosphatase (Rockland, PA, USA) for 12 hours at room temperature. The blot was developed with Nitro-blue tetrazolium chloride (Sigma) and 5-Bromo-4-chloro-3′-indolyphosphate p-toluidine (Sigma) as substrates to visualize the immunoreactive proteins.

3. Results

3.1. M3CLPY FRET biosensor as extracellular protease monitor

Limited information available regarding the types of proteases secreted by *Vibrio harveyi* and their target specificities posed limitations in designing a general FRET-based quantification assay for the bacterium's extracellular proteases. To overcome this constraint, we developed a novel FRET assay based on a large bacterial periplasmic binding protein (LuxP) fused to GFP variants. Periplasmic binding proteins fused to GFP variants have served well as intermolecular FRET biosensors in various studies (Fehr et al., 2002; Fehr et al., 2003; Rajamani et al., 2007). The LuxP structure without the signal peptide (342 residues), has a distance of 37 Å between the N- and C-terminal (Chen et al., 2002; Neiditch et al., 2005), was found useful for FRET measurements for BAI-2 binding (Rajamani et al., 2007). Mutations Q77A, S79A, and W82F were engineered into LuxP to generate M3LuxP with considerably lowered sensitivity for its