Switch of SpnR function from activating to inhibiting quorum sensing by its exogenous addition

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Abbreviations: QS, quorum sensing; AHL, *N*-acylhomoserine lactone; C6HSL, *N*-hexanoylhomoserine lactone; 3oxoC6HSL, *N*-(3-oxohexanoyl) homoserine lactone; MBP, maltose-binding protein; QCM, quartz crystal microbalance; CD, cyclodextrin; LB,

Luria-Bertani; DPA, 3,3'-dithiopropionic acid; NHS, *N*-hydroxysuccinimide; EDC,

N-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, IPTG,

isopropyl- β -D(-)-thiogalactopyranoside

Abstract

The opportunistic human pathogen *Serratia marcescens* AS-1 produces the *N*-hexanoylhomoserine lactone (C6HSL) receptor SpnR, a homologue of LuxR from *Vibrio fischeri*, which activates *pig* clusters to produce the antibacterial prodigiosin. In this study, we attempted to artificially regulate quorum sensing (QS) by changing the role of SpnR in *N*-acylhomoserine lactone (AHL)-mediated QS. SpnR was obtained as a fusion protein tagged with maltose-binding protein (MBP) from overexpression in *Escherichia coli*, and its specific affinity to C6HSL was demonstrated by quartz crystal microbalance analysis and AHL-bioassay with *Chromobacterium violaceum* CV026. Prodigiosin production was effectively inhibited by externally added MBP-SpnR in both wild-type AS-1 and the AHL synthase-defective mutant AS-1($\Delta spnI$). For the mutant, the induced amount of prodigiosin was drastically reduced to approximately 4% with the addition of 18 μ M MBP-SpnR to the liquid medium, indicating 81% trapping of C6HSL. A system for inhibiting QS can be constructed by adding exogenous AHL receptor to the culture broth to keep the concentration of free AHL low, whereas intracellular SpnR naturally functions as the activator in response to QS.

Keywords: Quorum sensing; LuxR family protein; Quorum quenching; *N*-acylhomoserine lactone

Introduction

The virulence expression of some opportunistic pathogens can be regulated by quorum sensing (QS), wherein activation is dependent on the concentration of a signal around cells [1,2]. QS is a cell-density-dependent system for the expression of target genes because the diffusible QS signal enables local accumulation through a cell membrane [3-5]. Various gram-negative bacteria produce *N*-acylhomoserine lactone (AHL) as the common QS signal, whereas QS activation for some gram-positive bacteria is triggered by increasing peptide concentrations [6,7]. The AHL complex with its intracellular receptor is stabilized by high AHL concentrations, and the complex interacts with a promoter region to activate transcription of the target gene. In many cases, receptors in bacterial cells exhibiting AHL-mediated QS are homologous to LuxR in *Vibrio fischeri*, which specifically recognizes *N*-(3-oxohexanoyl) homoserine lactone (3oxoC6HSL) [8-10]. In this study, we constructed a QS regulatory system based on the AHL trap using a LuxR homologue. Genetically engineered AHL receptor was added outside of cells to remove AHLs and inhibit QS.

As a model bacterium, *Serratia marcescens* AS-1 exhibits *N*-hexanoylhomoserine lactone (C6HSL)-mediated QS for the production of antibacterial prodigiosin, which is visualized as a red color [11,12]. The LuxR homologue SpnR, which possesses binding sites for C6HSL and the prodigiosin biosynthesis gene (*pig*) cluster promoter at the N- and C-termini, respectively, can form a complex with C6HSL to enhance QS. Intracellular SpnR natively

operates as a transcriptional activator of *pig* clusters, leading to the biosynthesis of prodigiosin synthase [13] upon formation of a complex with AHL (Fig. 1). Extracellular addition of SpnR in the culture broth is thus expected to effectively remove diffusible AHL molecules from the surrounding cells based on the high-affinity interaction. Thus, we overexpressed maltose-binding protein (MBP)-tagged SpnR (MBP-SpnR) in *Escherichia coli* and purified the fusion protein for application as a matrix with molecular selectivity [14].

Artificial reduction of the AHL concentration allows maintenance of the inactive state of QS even after reaching a quorum with respect to cell number. Previous studies have demonstrated that the amphiphilic AHL could be collected on cyclodextrin (CD) with hydrophobic cavities [15-17], carboxylate polymers copolymerized with hydrophobic domains [18,19], and self-assembled micelles of the block polymers containing hydrophilic and hydrophobic domains [20]. However, to the best of our knowledge, no report of the use of engineered natural products to inhibit QS by trapping AHL is available. Here, we demonstrated a method for changing the role of SpnR in AHL-mediated QS from activation to inhibition as a novel system for the artificial regulation of QS.

Materials and methods

Materials

3,3'-Dithiopropionic acid (DPA) and *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), isopropyl- β -D(-)-thiogalactopyranoside (IPTG), and ampicillin sodium were purchased from Wako Pure Chemical. All reagents for capillary gel electrophoresis including protein standards (SDS-MW analysis kit) were purchased from Sciex.

Purification of MBP-tagged SpnR

SpnR (28 kDa) derived from *S. marcescens* AS-1 was overexpressed as a fusion protein with an MBP tag in *E. coli* DH5 α (pMAL-*spnR*) grown in Luria-Bertani (LB) medium containing ampicillin and IPTG. After 24 h of culture, the cell pellet obtained by centrifugation was re-suspended in lysis solution (Bugbuster, Novagen) followed by repeated sonication with cooling. After centrifugation ($10,000 \times g$, 30 min), the supernatant was filtered with a 0.45- μ m cellulose ether membrane. The filtrate was injected into an amylose resin column (MBPTrap HP, GE Healthcare Life Sciences) on a chromatography system (ÄKTAprime plus, GE Healthcare Life Sciences) with Tris-HCl (pH 7.8) as the mobile phase. Protein adsorbed via affinity between amylose and MBP was eluted with 10 mM maltose solution, and the eluent fractions were collected in microtubes while monitoring the absorbance at 280 nm. A single peak appeared on elution, and the protein-containing fraction was collected. The concentration of the obtained MBP-SpnR solution was determined by

Lowry protein assay. Briefly, the sample solution was mixed with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in sodium tartrate solution. After mixing with Folin-Ciocalteu's phenol reagent and incubating for 30 min, the absorbance at 780 nm was measured on an ultraviolet–visible spectrophotometer (V-550DS, JASCO). The sample concentration was calculated from a standard curve constructed with bovine serum albumin. As a negative control, an MBP-LacZ α fusion protein (50.8 kDa) was purified from *E. coli* DH5 α harboring pMAL-c2X by the same method [21, 22].

Purification of the expressed MBP-SpnR and MBP- LacZ α was confirmed by capillary gel electrophoresis based on molecular weight analysis using sodium dodecyl sulfate (Sciex, PA 800 plus). Photodiode array detector analysis was carried out using a bare fused-silica capillary (inner diameter: 50 μm) with an effective length of 20.2 cm from the inlet to the detector window. The pretreated sample solution was injected into the capillary at 5.0 kV and migrated under 15.0 kV applied voltage. The peaks of the separated protein were detected by measuring the absorbance at 220 nm.

*Inhibitory effects on QS in *S. marcescens* AS-1*

As the model bacterium producing the LuxR family protein, *S. marcescens* AS-1 was grown in LB medium at 25 °C. After 1% inoculation with the pre-culture broth, the main culture was shaken in the presence of the desired concentration of MBP-SpnR (0–22.2 μM) at

25 °C for 18 h. During culture, the broth turned red as the prodigiosin production increased.

After centrifugation ($10,000 \times g$, 5 min), the cell pellet was re-suspended in 4% (v/v)

hydrochloric acid ethanol solution to extract the prodigiosin [13]. The absorbance at 534 nm

(A_{534}) of the supernatant was measured by UV-Vis spectrophotometer (V-550DS, JASCO)

after centrifuging again. The amount of prodigiosin produced per cell unit was calculated as

the ratio of A_{534} and the turbidity of the culture broth (OD_{600}). The relative prodigiosin

production was evaluated by normalization with the A_{534}/OD_{600} of the control without added

MBP-SpnR. The main culture was also performed with 2.2 or 11 μ M MBP-LacZ α as the

negative control at 25 °C for 18 h.

*AHL bioassay with *Chromobacterium violaceum* CV026*

Uptake of AHL by MBP-SpnR was studied using an AHL synthase-defective mutant

Chromobacterium violaceum CV026 [23,24]. Violacein, which exhibits a purple color, is

induced by the extracellular addition of short-chain AHLs (C4–C8) through the activation of

QS [25]. The pre-cultured medium of the CV026 strain for 10 h at 30 °C was mixed with 100

mL of fresh LB agar medium at the sol state as 1% inoculation. The pre-gel mixture was

poured into Petri dishes, and the agar gel plates for dispersing CV026 cells were prepared by

cooling. For C6HSL uptake by MBP-SpnR, a mixture containing 1 μ M C6HSL and 200 μ M

MBP-SpnR was incubated at 25 °C for 15 min. The aqueous solutions (20 μ L) of the mixture

and 1 μM C6HSL as the control were spotted onto a paper disk (ϕ : 8 mm) placed on the CV026 plate. Violacein production was induced for 15 h at 29 °C. The diameter of the purple spot is correlated with the concentration of C6HSL, which can be incorporated into the CV026 cell by self-diffusion.

*Effects of added C6HSL on QS in *S. marcescens* AS-1(ΔspnI)*

C6HSL added to the culture medium could induce QS-dependent prodigiosin production in the AHL synthase-defective mutant *S. marcescens* AS-1(ΔspnI), which is incapable of activating QS independently. The AS-1(ΔspnI) strain was cultured under the same conditions as AS-1 with the addition of the desired concentration (0–10 μM) of C6HSL during the main culture. Prodigiosin production per cell unit (A_{534}/OD_{600}) was evaluated as the degree of QS activation due to the external addition of C6HSL. The A_{534}/OD_{600} after the 15-h main culture was plotted against the initial concentration of C6HSL to construct a calibration curve.

Effects of added MBP-SpnR on prodigiosin induction in AS-1(ΔspnI)

The effects of adding MBP-SpnR on prodigiosin production were investigated under the QS-activated condition in the AS-1(ΔspnI) strain with the desired initial concentration of C6HSL. The pre-culture was inoculated in 4 mL of fresh LB medium containing 2 μM C6HSL and 0–18 μM MBP-SpnR. Prodigiosin production was allowed to be induced by only

C6HSL at the determined concentration, whereas QS in wild-type AS-1 was activated by both C6HSL and 3oxoC6HSL with varying concentrations.

The A_{534}/OD_{600} value determined in the presence of MBP-SpnR was extrapolated to the calibration curve to estimate the apparent initial C6HSL concentration.

QCM analysis

The molecular interaction between MBP-SpnR and immobilized C6HSL was analyzed by quartz crystal microbalance (QCM) under the thickness shear mode of vibration induced by AC voltage (Affinix QN Pro, ULVAC). A quartz plate (27 MHz, AT cut) sandwiched by two gold electrodes was placed in the bottom of the cup-shaped cell. The electrode was modified with C6HSL as reported previously (Fig. 2a) [14]. Briefly, activated esters of the carboxylate groups on a DPA self-assembled monolayer were prepared by reaction with EDC and NHS. The homoserine lactone moiety was introduced on the electrode by coupling C6HSL with the amino groups. After stabilizing the oscillation in phosphate buffer solution (pH 7.0) at 25 °C, 10 μ L of 0.88 μ M MBP-SpnR sample solution was gently injected into the QCM cell under peltier temperature control (20 °C; Fig. 2b). The time course of the conductance was followed by network analyzer analysis. The mass load from the C6HSL and MBP-SpnR interaction can reduce the specific value of F_2 , the higher frequency at half of the conductance maximum. The uptake mass of MBP-SpnR can be evaluated more accurately

(0.62 ng cm⁻²) from ΔF_2 because this value is unaffected by the viscous load due to the interaction between the electrode surface and the solution [26,27]. To monitor the molecular recognition of the receptor, an aqueous solution of MBP-SpnR with or without thermal deactivation at 80 °C for 1 h was injected to the C6HSL-modified electrode.

Results and discussion

Specific interaction of C6HSL with the added MBP-SpnR

Specific complex formation between immobilized C6HSL and added MBP-SpnR was studied by measuring ΔF_2 with QCM analysis. The quartz plate coated with a gold electrode (4.9 mm²) was modified with C6HSL moieties (8.4 pmol; Fig. 2a). MBP-SpnR was purified by affinity chromatography with amylose resin, and its molecular weight, as determined by capillary gel electrophoresis, was consistent with the calculated value. The ΔF_2 value clearly decreased, indicating MBP-SpnR uptake onto the electrode (Fig. 2b). The mass increase on the electrode depends on the specific bioaffinity because the decrease in ΔF_2 was hardly observed after thermal denaturation of MBP-SpnR at 80 °C; the detection sensitivity of the QCM system is 30 pg/Hz.

In the AHL bioassay, the appearance of a purple color from violacein on agar plates containing CV026 cells showed the activation of QS after external addition of C6HSL (Fig. 3a). Addition of C6HSL mixed with MBP-SpnR (1:200) onto the agar plate did not induce

observable production of violacein. These results indicate that only a low concentration of C6HSL was available during the 15-h incubation, suggesting a strong interaction between C6HSL and MBP-SpnR. The sensitivity of the CV026 assay depends on the chemical structure of the acyl-chains; C6HSL is the natural AHL produced in CV026 cells and the most active agonist, whereas the agonist activity of 3oxoC6HSL in the AS-1 strain was approximately one-sixth that of C6HSL [25]. Both QCM analysis and the AHL bioassay revealed effective complex formation between MBP-SpnR and C6HSL.

Inhibitory effects of added MBP-SpnR on C6HSL-mediated prodigiosin production

The red pigment prodigiosin in wild-type *S. marcescens* AS-1 is induced by the activation of *pig* clusters via sufficient accumulation of AHL [11]. We added MBP-SpnR to the cell culture as an AHL trap to study the inhibitory effects on QS. Intracellularly accumulated prodigiosin was extracted from the cell pellet after centrifugation of the culture broth. The normalized relative prodigiosin production decreased with as the concentration of MBP-SpnR increased (Fig. 3b); no concomitant effect on cell growth was observed based on the OD_{600} . With the addition of 2.2 μ M MBP-SpnR, the amount of prodigiosin induced was half that of the control without any additives. α -CD, one of the AHL host molecules that was previously investigated, inhibited prodigiosin production in the AS-1 strain by half when added to the culture broth at 10 mM [16]. The greater inhibitory effect of MBP-SpnR on QS was

attributed to the higher affinity of the native receptor for C6HSL. The additive MBP-SpnR specifically interacted with C6HSL, as evidenced by the fact that the relative prodigiosin production and the OD_{600} were 0.94 and 6.30, respectively, after 18 h of culture in the presence of 2.2 μ M MBP-LacZ α (Fig. 3c).

Inhibitory effects of added MBP-SpnR on QS induced by exogenous C6HSL

The degree of QS activation in the AS-1(Δ *spnI*) strain with the addition of exogenous C6HSL to the liquid culture medium was investigated (Fig. 4a). The prodigiosin production per cell unit (A_{534}/OD_{600}) after 15 h of culture increased as the C6HSL concentration was increased and approached a constant value, indicating an increasing degree of QS activation.

The leveling off behavior is plausible for QS-dependent prodigiosin production, which is activated above a threshold concentration of C6HSL. Because the AS-1(Δ *spnI*) strain produces no endogenous C6HSL, prodigiosin production is induced only by the known concentration of added C6HSL. This system allows analysis of both QS inhibition and the C6HSL trap efficiency of the added MBP-SpnR in real culture broth. The relative prodigiosin production level was evaluated at different MBP-SpnR concentrations with 2 μ M C6HSL.

The proportion of AHL trapped by MBP-SpnR was calculated by extrapolation of the A_{534}/OD_{600} values to the calibration curve (Fig. 4a). Adding 18 μ M MBP-SpnR inhibited QS-mediated prodigiosin production to approximately 4% by removing 81% of added

C6HSL (Fig. 4b). These results suggest that MBP-SpnR forms a complex with C6HSL in LB medium and that this complex is stable during the shaking culture.

Previously, the stability constant (K) of the MBP-SpnR–C6HSL complex was reported as $K = (2 \pm 1) \times 10^6 \text{ dm}^3/\text{mol}$ [14]. After the cell number reaches quorum, the C6HSL concentration sufficiently increases above its threshold to stably form the C6HSL–SpnR complex, which induces the interaction between SpnR and the *pig* promoter. In the multistep QS process, SpnR acts as a transcriptional activator for the *pig* promoter, and its affinity for the promoter changes upon complexing with C6HSL.

In conclusion, in this study, we demonstrated that the function of SpnR could be switched by adding the protein to the cell culture. Exogenous SpnR has high affinity for C6HSL and functions as a host matrix to sequester secreted C6HSL and inhibit QS. In addition, it inhibits QS to a greater extent than CD, which includes the hydrophobic acyl chain of C6HSL in aqueous media. Thus, the AHL receptor can be used to artificially regulate AHL-mediated QS by changing its role from activation to inhibition.

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Figure Legends

Figure 1. Schematic representation of the molecular interactions among N-hexanoylhomoserine lactone (C6HSL), SpnR, and the *pig* promoter for prodigiosin production in *S. marcescens* AS-1.

Figure 2. Quartz crystal microbalance (QCM) analysis of the specific interaction between MBP-SpnR and immobilized C6HSL. (a) Chemical structure of the C6HSL-modified QCM electrode. (b) Time course of frequency change (ΔF_2) in response to binding of MBP-SpnR to a C6HSL-immobilized electrode. An aqueous solution of MBP-SpnR with or without thermal deactivation at 80 °C for 1 h was injected at $t = 0$.

Figure 3. Inhibitory effects of externally added MBP-SpnR on quorum sensing (QS)-mediated prodigiosin production in wild-type AS-1. (a) Demonstration of MBP-SpnR affinity with C6HSL using AHL-reporter CV026. Left: control, 1 μ M C6HSL; right: 200 μ M MBP-SpnR and 1 μ M C6HSL. No observable purple color was produced with addition of the C6HSL trap with MBP-SpnR. (b) Dependence of relative prodigiosin production and turbidity of the culture broth (OD_{600}) on MBP-SpnR concentration. Reduced effects of added MBP-SpnR on prodigiosin production were evaluated as the index of QS inhibition. (c) Dependence of relative prodigiosin production and the OD_{600} on the MBP-LacZ α

concentration.

Figure 4. Activation and inhibition of C6HSL-mediated QS in *S. marcescens* AS-1($\Delta spnI$).

(a) Calibration curve for the degree of C6HSL-activated QS with different initial C6HSL concentrations. The AS-1($\Delta spnI$) strain was cultured in Luria-Bertani (LB) medium containing C6HSL at 25 °C for 18 h. (b) Reduced effects of added MBP-SpnR on prodigiosin production with 2 μ M C6HSL. The AHL trap percentage was estimated from the calibration curve.

Figure1

Figure2

Figure3

Figure4