Development of a reversible regulatory system for gene expression in the cyanobacterium *Synechocystis* sp. PCC 6803 by quorum-sensing machinery from marine bacteria

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Abstract

Histidine kinases are common sensory proteins used to detect environmental changes in bacteria. They respond to specific stimuli via a signal-input domain and alters gene expression through a cognate response regulator. The modulation/control of transcriptional regulation in cyanobacteria is important to reinforce the production of useful target compounds via photosynthesis without altering the growth profiles. For instance, heavy metal ions (Ni^{2+} and Cu^{2+}), chemical inducers (IPTG), and a volatile compound (toluene) have been previously applied to regulate gene expression in cyanobacteria. However, most systems/regulators are only able to regulate gene expression once because it is impossible to eliminate them from the medium. To construct a reversible regulation system, a chimeric sensor, VanN_SphS, was developed by fusing the signal input domain of a quorum-sensing (QS) sensor, VanN, from *Vibrio anguillarum*, responding *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), with the kinase domain of SphS, a phosphate-deficiency sensor from the cyanobacterium *Synechocystis* sp. PCC 6803. After expression of the chimeric sensor in *Synechocystis* cells, responses to the various *N*-acyl-homoserine-lactones (AHLs) were evaluated by measuring the alkaline phosphatase (AP) activity, which is regulated by SphS. VanN_SphS responded only to OHC6-HSL and repressed AP activity. Then, the coexpression of the AHLs-degradation enzyme, Aii20J, a lactonase from *Tenacibaculum* sp. 20J, resumed the activity. This is the first report on the use of AHL-mediated transcriptional regulation in *Synechocystis*, which could be used in the future for the controlled production of useful compounds in the cyanobacterium.

Keywords

Histidine kinase; Chimeric sensor; reversible gene regulation; AHLs; VanN; SphS

1. Introduction

Metabolic engineering is a promising technique to produce valuable products by living organisms. However, the adequate usage of all the cellular resources for the synthesis of targeted products and the increased metabolic burden on the cells are challenges that remain to be addressed (Stephens et al. 2019). Because of the accumulation of genomic information and the development of genetic modification tools, the application of heterotrophic microorganisms is more prosperous than that of the photosynthetic organisms. However, the former needs organic compounds (e.g., low-molecular-weight sugars, organic acids, and alcohols) to be added to the extracellular matrix for growth. On the contrary, the latter can convert inorganic materials, including CO_2, into the target products using light energy via photosynthesis. Therefore, the application of photosynthetic organisms may have practical carbon usage. The production of targeted compounds from low-molecular-weight fixed carbon or CO_2 requires the regulation of the distribution of carbon between the synthesis of components of cellular bricks and the targeted compounds. An artificial gene regulatory system may be an effective approach in the regulation of cellular metabolism switching from the growth phase to the production phase of the targeted compounds (Dexter and Fu 2009; Kato et al. 2016; Inaba et al. 2018).

In microorganisms, extracellular environmental stimuli can be perceived and responded through a two-component signal transduction system, composed of a stimulus specific sensory protein, histidine kinase (HK), and a response regulator (RR) protein. An environmental signal perceived by the signal-input domain of HK located at the N-terminal results in an alteration in autophosphorylation status at the conserved histidine residue that acts as a phospho-acceptor/donor and transfers the phosphoryl group to the aspartate residue of the RR protein (Grimshaw et al. 1998; Stock et al. 2000; Zschiedrich et al. 2016). The expression of certain regulon genes is controlled according to the phosphorylation status of the RR (Koretke et al. 2000; Stock et al. 2000). Various functions including development, motility, virulence, and metabolism, are regulated by two-component signaling systems in microorganisms. Previously, gene regulation through chimeric HK and the application of exogenous stimuli has been demonstrated. The use of a chimeric HK, Tar-EnvZ, which can respond to a chemical stimulus, aspartate, for enzymatic regulation, has been demonstrated in *Escherichia coli* (Yoshida et al. 2007). To promote the expression of the *gfp* and *ompC* genes to detect fumarate concentrations, Ganesh et al. (2013) developed a chimeric sensor, DcuSZ, by using the sensory HK, DcuS, with the EnvZ domain from *E. coli.* They found that their chimeric sensor was able to detect other C4-dicarboxylates along with fumarate in the media. In *E. coli*, the transgenic production of phycocyanobilin (PCB) has been regulated through the development of a photo-

perception system using the two-component CcaS-CcaR from *Nostoc punctiforme* (Sugie et al. 2016) which allowed gene regulation in an ON/OFF switch manner by using specific light wavelengths (Hori et al. 2017).

The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) adapts to environmental changes by regulating its cellular behavior and gene expression through two-component regulatory systems (Hoch 2000; Alves and Savageau 2003; Marin et al. 2003; Hsiao et al. 2004; Suzuki et al. 2004). The presence of 47 genes for HKs and 44 genes for RRs in the genome of *Synechocystis* has been confirmed by genome sequence analysis, where each HK identifies a variety of environmental signals (Kaneko et al. 1996; Mizuno 1997; Kaneko et al. 2003). Among the various HKs present in *Synechocystis*, Hik7 (SphS) has been identified to perceive a signal for phosphate deficiency in the environment (Aiba et al. 1993; Nagaya et al. 1994; Hirani et al. 2001; Kimura et al. 2009). To acclimatize to phosphate-limiting environments, the HK SphS (sll0337), and its cognate RR, SphR (slr0081), regulate the expression of the *pho* regulon under phosphate starvation. The up-regulation of the *pho* regulon can be detected through the measurement of the activity of the alkaline phosphatase (AP) encoded by the *phoA* gene in the *Synechocystis* (Ray et al. 1991; Aiba et al. 1993; Hirani et al. 2001). AP activity in the microorganisms is localized in the periplasmic space and releases the orthophosphate from the phosphor-esters, which can penetrate through the outer membrane and peptide-glycan layer, therefore contributing to supply phosphate to the cells under the phosphate-limiting conditions.

The use of the kinase domain of SphS, a phosphate-deficiency sensor, for the development of chimeric sensors in *Synechocystis* has been demonstrated in previous studies. Shimura et al. (2012) constructed a chimeric HK, Hik33n_SphSc, by fusing the signal input domain of the HK, Hik33, and the kinase domain of SphS. The response of this chimeric Hik33n_SphSc to several stimuli was evaluated by monitoring the regulation of the *phoA* gene, responsible for AP activity which allowed the characterization of the functional domains for the perception of the stimuli by the deletion and mutation of the domains in the chimeric HKs. To evaluate the function of an uncharacterized HK Hik2, conserved in all the cyanobacterial genome, the chimeric sensor Hik2n_Hik7c was constructed by joining the N-terminal region of Hik2 and the C-terminal of the phosphate-deficiency sensor domain, SphS. The chimeric sensor responds to the NaCl concentrations, actually Cl^-, in the media, and induces AP activity (Kotajima et al. 2014). The chimeric sensor TodS_SphS, developed by fusing the signal input domain of TodS, a toluene-sensor from *Pseudomonas putida* F1, and the kinase domain of SphS shows the successful regulation of gene expression for the AP assay via a volatile chemical, toluene, in the cyanobacterium *Synechocystis* (Inaba et al. 2018). They also showed that the removal of the volatile inducer, toluene, in their system, enabling the ON/OFF regulation of the chimeric HK.

Quorum sensing (QS) is a cellular communication process based on extracellular signaling molecules, autoinducers, that allows coordinated regulation of gene expression in bacterial populations. Depending on the number of cells, the autoinducers produced accumulate in the medium environment and alter the bacterial behavior via gene expression regulation. Different types of QS signals and sensors have been described, and a two-component system also contributes to regulating gene expression in some cases. *N*-acyl-homoserine lactones (AHLs) are the most common autoinducers used by gram-negative bacteria to induce the QS process (Bassler and Losick 2006). A membrane-bound sensor kinase, LuxN, from the marine bacterium *Vibrio harveyi*, detects the AHL, *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL), for the regulation of gene expression (Freeman et al. 2000; Henke and Bassler 2004; Timmen et al. 2006; Jung et al. 2007). The sensory domain of VanN, a homolog of LuxN, from the pathogenic marine gram-negative bacterium *Vibrio anguillarum*, can respond to *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), which is produced by the AHL synthase encoded by the *vanM* gene (Milton et al. 2001; Buchholtz et al. 2006). Both LuxN and VanN possess nine transmembrane (TM) helices and have similar structures, with 849 and 859 amino acids, respectively (Bonneau 2008).

The process contrary to QS, involving the enzymatic degradation of the autoinducers, is also known as quorum quenching (QQ). Although several types of QQ enzymes exist (Romero et al. 2015), AHL-lactonases are commonly used as the QQ enzyme, which are found to have wider substrate spectra and a more effective degradation of AHLs, including, short and long chain AHLs such as *N*-butanoyl-L-homoserine lactone (C4-HSL), *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), and *N*-oxododecanoyl-L-homoserine (C12-HSL) (Dong et al. 2001; Ulrich 2004; Mayer et al. 2015). AiiA was the first AHL-lactonase identified in *Bacillus* sp. strain 240B1, and its expression in *Erwinia carotovora* SCG1 was found to degrade AHLs and to reduce the pathogenicity of this bacterium in various crops (Dong et al. 2000). Thus far, although many lactonases have been identified in the genus *Bacillus* sp. (Liu et al. 2008; Momb et al. 2008; Chen et al. 2010), the marine bacterium *Tenacibaculum* sp. 20J presents a wider range of QQ activity compared to *Bacillus* strains (Romero et al. 2011) and shows constitutive and cell-bound QQ activity by degrading the AHLs produced by the fish pathogen *Edwarsiella tarda* (Romero et al. 2014). Mayer et al. (2015) characterized an AHL-lactonase enzyme, Aii20J, from *Tenacibaculum* sp. 20J, with a wide spectrum and high specific AHL quenching activity and resistance to high temperature, alkalinity, and protease degradation.

In the present study, we constructed two novel chimeric sensors by fusing the QS sensory domain of the kinases, LuxN and VanN, with the kinase domain of SphS, to detect their response to exogenously applied AHLs

for the regulation of AP activity in *Synechocystis*. In combination with the QQ enzyme, Aii20J, we developed a novel reversible regulatory system in cyanobacterial cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used as a wild-type strain (WT) in this study. ΔSphS, a strain with SphS deletion (Kimura et al. 2009), and three *Synechocystis* transformants, LuxN_SphS, VanN_SphS, and VanN_SphS::Aii20J (in this study) were grown in BG-11 medium (Stanier et al. 1971). The BG-11 medium was buffered with 20 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH (pH 6.5). The temperature for all the cultures was maintained at 34°C with continuous illumination at 70 µmol photons m^{-2} s^{-1} intensity by incandescent lamps and aeration of 1% (*v/v*) CO_2-enriched air during the experiments (Wada and Murata 1989). Solidified BG-11 medium with 1.5% (*w/v*) Bacto agar (Japan Becton Dickinson, Tokyo, Japan) supplemented with antibiotics spectinomycin dihydrochloride salt (25 µg mL^{-1}) and chloramphenicol (25 µg mL^{-1}) was used to maintain the transformants LuxN_SphS, VanN_SphS, and VanN_SphS::Aii20J, respectively. The growth of the cells was monitored by measuring the optical density of the culture at 730 nm (OD_{730}) using a spectrophotometer UV-1900 (Shimadzu, Kyoto, Japan). *Chromobacterium violaceum* CV026, an AHL biosensor strain, was cultured on lysogeny broth (LB) medium at 30°C and used for the AHL degradation plate bioassays (McClean et al. 1997).

Plasmids were constructed using competent *E. coli* JM109 cells (Takara Bio, Ohtsu, Japan) as a host cell via the heat shock method. LB medium was used to screen and maintain these *E. coli* cells harboring the engineered plasmids at 37°C and shaking at 180 rpm. Suitable antibiotics (50 µg mL^{-1} of sodium ampicillin, spectinomycin dihydrochloride salt, and chloramphenicol) were supplied in LB when required.

2.2. AHL-sensing chimeric histidine kinases (HK)

We identified the canonical QS-sensors LuxN and VanN from *V. harveyi* BB120 and *V. anguillarum* 90-11-287, respectively, to be used as a signal sensor domain for the recognition of AHLs or autoinducers. The QS sensor LuxN recognizes the cognate signal OHC4-HSL, whereas its homolog VanN responds to OHC6-HSL (Buchholtz

et al. 2006; Swem et al. 2008). It was verified that these AHLs have no antibiotic effect against *Synechocystis* (Kaufmann et al. 2005) and the stability of the AHLs in the cells and culture medium of the cyanobacteria was also analyzed. Among the available HKs to use as a kinase domain, we selected SphS (Hik7, sll0337), a Pi-deficiency sensory kinase from *Synechocystis*, involved in the expression of AP.

2.3. Construction of chimeric sensors

After selecting the HKs for the present study, we amplified the DNA fragments corresponding to the signal-input domains of LuxN and VanN and integrated them into the SphS-expression vector (pSK05ΔPAS; Kimura et al., 2009) with the substitution of the signal input domain of SphS using In-Fusion® HD cloning Kit (Takara Bio) to express the two chimeric sensors, LuxN_SphS and VanN_SphS. The amino acid regions of LuxN (M1-R460) and VanN (M1-C460) were connected to the C-terminus of SphS (G197-P430), respectively. Briefly, the DNA fragments, corresponding to the signal input domains of LuxN and VanN, were amplified by polymerase chain reaction (PCR) using the genomic DNA of the respective *Vibrio* species as the templates and the primers LuxN_F and LuxN_R, VanN_F, and VanN_R (Supplementary Table 1). We also amplified the vector backbone of pSK05ΔPAS, including the region of approximately 1-kbp upstream and downstream of the kinase domain of the SphS gene, the spectinomycin resistance gene cassette, and the pUC vector (Kimura et al. 2009). The synthetic plasmids, pSK05LuxN::SphS and pSK05VanN::SphS, were transformed into the *Synechocystis* strain ΔSphS, in which the coding sequence of the *SphS* gene was deleted. The chimeric genes, LuxN_SphS and VanN_SphS, were introduced into the *Synechocystis* chromosome by double homologous recombination (Williams 1988; Shimura et al. 2012; Kotajima et al. 2014). For the transformation of the cyanobacterium, ΔSphS cells were cultivated in BG-11 medium until the logarithmic growth phase. After centrifugation, the cell pellet was resuspended in a one-tenth volume of BG-11 medium together with the plasmid DNA and incubated overnight under dim light with shaking at 30°C. The transformed colonies with pSK05LuxN::SphS and pSK05VanN::SphS plasmids were screened by the resistance of spectinomycin grown on the agar-solidified BG-11 medium containing 5 μg mL^{-1} spectinomycin dihydrochloride. The spectinomycin-resistant colonies were transferred and maintained on agar-solidified BG-11 plates with a higher concentration of spectinomycin dihydrochloride salt (25 μg mL^{-1}). The functionality of these newly developed chimeric sensor proteins in *Synechocystis* was assessed with respect to the response of AP activity to the addition of AHLs.

2.4. Expression of the quorum-quenching lactonase (Aii20J)

Synechocystis does not possess a native system to degrade AHLs. Therefore, we attempted to express Aii20J, an AHL-lactonase from the marine bacterium *Tenacibaculum* sp. 20J (Mayer et al., 2015), in *Synechocystis* cells harboring the VanN_SphS chimeric sensor. The codon usage of the sequence of Aii20J (Supplementary Fig. 1) was optimized for the expression in *Synechocystis* by Codon Optimization OnLine (COOL) (https://cool.syncti.org) and synthesized by Eurofins Genomics (Tokyo, Japan). The DNA fragments for the coding sequence of Aii20J and pTHCT2031V (Ishizuka et al. 2006), which harbors homologous sequences for the neutral site and the p*Trc* promoter and chloramphenicol resistance gene, were amplified by PCR using the primers βLAC_F and βLAC_R, pTCHT_slr2031_F and pTCHT_slr2031_R, respectively. The resultant fragments were conjugated using an In-Fusion kit to construct plasmid pTHCT2031V::Aii20J and introduced into *Synechocystis* cells harboring the VanN_SphS chimeric sensor by homologous recombination at the neutral site in the cyanobacterium chromosome (slr2031). The resulting strain was named VanN_SphS::Aii20J.

2.5. Acyl homoserine lactones (AHLs)

We examined the effect of various AHLs with different lengths of carbon chains and with different functional groups on the chimeric sensors. The AHLs used in this study were *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL racemic), *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL), *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), *N*-(*β*-ketocaproyl)-L-homoserine lactone (OC6-HSL), and *N*-dodecanoyl-L-homoserine lactone (C12-HSL). The AHLs were purchased from Sigma-Aldrich (Japan). These AHLs were dissolved in acetonitrile to prepare a 10 mM stock solution and administered into the culture medium to dilute 1:1000 (*v/v*) to obtain a working solution of 10 μM.

2.6. AHL degradation assay

The quorum quenching in the VanN_SphS::Aii20J strain was detected by the *C. violaceum* CV026 bioassay (Romero et al. 2010). The cells of *C. violaceum* CV026 respond to various AHLs but not OHC6-HSL (McClean et al 1997), which was the active AHL in the case of VanN_SphS. Thus, in order to detect the expression Aii20J in the mutant, we determined the degradation activity against *N*-hexanoyl-L-homoserine lactone (C6-HSL), instead

of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL), in the cultures of VanN_SphS and VanN_SphS::Aii20J. Briefly, 10 mM stock solution of C6-HSL in acetonitrile was added to the VanN_SphS and VanN_SphS::Aii20J aeration cultures to achieve a final concentration of 10 μM in the cultures. A time-course experiment was followed for 48 h in which each sample was taken at an interval of 6 h. Cells were removed by centrifugation at 5000 × *g* for 3 min and the presence of AHLs in the supernatant was analyzed by the *C. violaceum*-based solid-plate bioassay. One mL of CV026 concentrated overnight culture grown at 30°C was mixed with 4–5 mL of liquid soft LB (0.8 % (*w/v*) Bacto-agar). This mixture was then added to the LB plates to create a homogenous layer. The plates were allowed to solidify for 10–15 min. After solidification, a sterilized glass tube (ϕ = 6 mm) was used to create wells, each of which was filled with 100 μL of the cell-free culture medium. After incubation for 12 h at 30°C, the generation of the purple pigment (Violacein) halo by C6-HSL was observed. The sizes of the purple halos were determined and the concentration of the AHL was estimated by comparison with those from the known concentrations.

2.7. Alkaline phosphatase (AP) assay

The activity of AP was assayed by detecting the rate of degradation of *p*-nitrophenyl phosphate (PNPP) (Aiba et al. 1993). AP is localized in the periplasmic space of cyanobacterial cells. PNPP penetrates the periplasmic space through the outer membrane and is easily separated by the enzyme after the precipitation of the cells by centrifugation. Because its expression was maintained under sufficient illumination, the activity gradually decreased due to the limitation of illumination during the culture as a result of self-shading (Asada et al. 2019).

The LuxN_SphS and VanN_SphS strains were inoculated at an initial OD_{730} of 0.2 in 50 mL fresh BG-11 medium. The cultures were grown with or without AHL addition. AP activity was measured from the harvested cells at different time intervals.

For the AP recovery experiment, increasing the culturing time resulted in denser cultures, making light a limiting factor for the recovery of AP activity. Hence, a unique growth technique was used to keep the culture's optical density OD_{730} of 1.0 in every 24 h interval by using the following formula:

$$X = \left(r - \frac{r}{n}\right) \text{ x C (mL)} = \left(1.0 - \frac{1.0}{n}\right) \text{ x C (mL)}$$

where,

X = volume of culture to be withdrawn and centrifuged

r = the required optical density {OD_{730} = 1.0}

n = the change in optical density every 24 h

C= amount of the culture remaining in the tubes after every 24 h of aeration in mL

The specific culture volume obtained after the calculation was centrifuged (5000 × *g*) for 3 min and the resulting supernatant was returned to the original culture tubes to maintain the same OHC6-HSL concentration and OD_{730} = 1.0. The cell pellet obtained from centrifugation was resuspended with the same amount of new BG-11 medium as obtained from the calculation. AP was measured from the cell pellets.

To measure the AP assay for all the above experiments, 700 µL of 285 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH buffer (pH 9.5) was mixed with 200 µL of culture at 35°C on a heat block. The AP assay reaction was started by adding 100 µL of 36 mM PNPP in the cell suspension mixture and stopped by the addition of 100 µL of 4 M NaOH at 5 and 20 min time intervals in separate tubes. After centrifugation (10000 × *g*, 1 min), the absorbance of the supernatant was measured at 397 nm using a UV-VIS Spectrometer (UV-1900). The standard curve of *p*-nitrophenol in CAPS-NaOH buffer (pH 9.5) was used to determine the amount of *p*-nitrophenol (µmol mL^{-1}). To measure the Chl*a* (mg mL^{-1}) amount, 500 µL of cell suspension was centrifuged (18000 × *g*, 1 min, 4°C), and the resulting cell pellet was resuspended in 900 µL of 90% methanol. After another round of centrifugation, the absorbance of the supernatant was measured at 665 nm (Tandeau de Marsac and Houmard 1988).

2.8. RNA extraction and measurement of phoA mRNA expression by real-time PCR

The relative expression of *pho*A mRNA was measured by real-time PCR. Briefly, the extraction and purification of the total RNAs were performed by Invitrogen TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, Tokyo, Japan) and RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively (Kotajima et al. 2014). VanN_SphS cultures with and without the addition of OHC6-HSL were used to extract total RNA. OHC6-HSL was added to the cells at a final concentration of 10 µM. The culture samples were taken at 10-min intervals until 1 h. For cell fixation, 50 mL cell culture was taken at each sampling point and mixed instantly with an equal volume of ice-cold 10% phenol/ethanol (*v/v*). After centrifuging this mixture at 2800 × *g* for 5 min at 4°C, the supernatant was discarded, leaving 500–1000 µL for the resuspension of the obtained cell pellet. The pellet was transferred into a 1.5 mL tube and centrifuged at 12000 × *g* for 3 min at 4°C, completely removing the supernatant. The cell pellet was then added to 0.5 g of zirconia beads (0.1 mm in diameter) and 1 mL of TRIzol. The mixture was vortexed for 5 min using MINI-BEADBEATER™ (BioSpec Products, Bartlesville, OK). Subsequently, for

the bilayer distribution step, 200 μL of iced chloroform was added. The samples were mixed vigorously by shaking, kept at room temperature for 5 min, and then centrifuged at 15000 × *g* for 20 min at 4°C. The resulting upper aqueous phase (400 μL) was transferred to a new tube and mixed with 300 μL of 100% ethanol. RNeasy Mini Kit was used to purify the obtained RNAs according to the supplier's manual (Qiagen).

From the 1 μg of purified total RNAs, complementary DNA (cDNA) was synthesized using a PrimeScript RT reagent Kit with a genomic DNA (gDNA) Eraser (Perfect Real Time) (Takara Bio). Real-time PCR was performed using a GOTaq qPCR Master Mix (Promega, Madison, WI) in a PikoReal 96 Real-Time PCR system (Thermo Fisher Scientific). The *phoA* gene expression levels were measured using the forward primer, PhoA_RT_F, and reverse primer, PhoA_RT_R, whereas a set of forward and reverse primers, rpnB_RT_F and rpnB_RT_R, respectively, were used to amplify the expression of the reference gene, *rnp*B, which is a functional RNA for Rnase P (Supplementary Table 1). PikoReal software version 2.2 (Thermo Fisher Scientific) was used to obtain the threshold cycle (C_T) values. The relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen (2001).

2.9. Statistical analysis

The statistical package R (version 3.6.1) (R Development Core Team 2011) was used for statistical analysis. The data were considered statistically significant at a probability level (p-value) of ≤ 0.05. A two-way analysis of variance (ANOVA) with interaction was performed for the time course AP activity experiments using different AHLs, AP recovery experiments, and the relative *phoA* mRNA expression, and Holm's sequential Bonferroni correction (Holm 1979) was performed as a post hoc analysis for significant pairwise comparisons of the data.

3. Results and discussion

3.1. Development of the chimeric sensors LuxN_SphS and VanN_SphS

In the present study, we developed a novel regulatory system for gene expression based on the QS sensory domains of LuxN, VanN, and the QS signaling molecules, AHLs. The LuxN and VanN sensory domains have 849 aa and 859 aa, respectively, with nine TM regions (Bonneau 2008; Milton et al. 2001). These TM regions were fused with the C-terminal kinase domain of SphS from *Synechocystis*, which is responsible for the expression of

AP under phosphate-deficient conditions to produce chimeric sensors LuxN_SphS and VanN_SphS. SphS possesses its own TM helices and the PAS domain, which are important for the function of the inorganic phosphate (Pi)-sensor and the kinase domain (Kimura et al., 2009). The substitution of the TM helices with another TM helix of the Ni-sensor from *Synechocystis* resumes the function of the Pi-sensor, but not the Ni-sensor. In this study, we substituted the signal input domain of SphS, including the TM helices and the PAS domain, with the TM regions of LuxN (M1-R460) and VanN (M1-C460), and connected them to the kinase domain of SphS (G197-P430), respectively (Fig. 1). After introducing the genes for these chimeric sensors in *Synechocystis* and confirming the insertion by sequencing, we studied the effect of exogenously applied AHLs by measuring the AP activity.

3.2. Effect of the various autoinducers (AHLs) on the chimeric sensors

AHLs consist of a homoserine lactone ring and acyl chain. The AHLs show differences in terms of the length of the acyl chain moiety, as well as a substitution at the position of carbon 3 (C3). Commonly, the acyl chain is 4–12 carbons in length; however, some AHLs have up to 14–18 carbons. The C3 position may be either unmodified or modified with a carboxyl or a hydroxyl group substituent (Marketon et al. 2002; Thiel et al. 2009). The AHLs used in these experiments were C6-HSL, C6-HSL racemic, OHC4-HSL, OHC6-HSL, OC6-HSL, and C12-HSL (Fig. 2).

AP activity assays were conducted with and without the addition of the above-mentioned AHL compounds in aerated cultures of LuxN_SphS and VanN_SphS strains until 2 d. In the case of LuxN_SphS, AP activity was negligible without the addition of AHLs and did not respond to any of the tested AHL compounds (Fig. 2a-f). In contrast, for the VanN_SphS cultures, AP activity was expressed without the addition of AHLs, indicating that the chimera VanN_SphS was able to phosphorylate the RR, SphR, without any AHLs. The AP activity in the cells with and without the addition of the AHL compounds was not significantly altered except for the administration of OHC6-HSL, namely *N*-3-hydroxyhexanoyl-L-homoserine lactone (Fig. 2a-f), the cognate signal for VanN. A two-tailed Student's *t*-test indicated that the repression of AP activity was significantly altered due to the addition of OHC6-HSL (Fig 2d).

The addition of OHC6-HSL to the VanN_SphS cultures resulted in a significant decrease in AP activity over time, and within 2 d, the AP activity reached almost zero. Until 2 d, in the VanN_SphS cells without the addition of OHC6-HSL, the AP activity was 1.20 ± 0.215 (μmol PNP mg^{-1}Chl*a* min^{-1}) and decreased significantly in cells containing exogenously applied OHC6-HSL, reaching 0.08 ± 0.009 (μmol PNP mg^{-1}Chl*a* min^{-1}) (Fig. 2d). Two-

way ANOVA implied that both AHL application (p = 0.000) and time (p = 0.000) had highly significant effects for the OHC6-HSL for the VanN_SphS chimeric sensor (Fig. 2d). The interaction (AHL × time) until 2 d (p = 0.000) was also highly significant. Moreover, post-hoc analysis using Holm's sequential Bonferroni correction (Holm 1979) suggested that the addition of OHC6-HSL to VanN_SphS cells significantly decreased (p = 0.0003) the AP activity compared to VanN_SphS cells without the addition of OHC6-HSL (Fig. 2d).

These results indicated that the chimeric sensor, LuxN_SphS, did not function in our case because it could not respond to any of the applied AHLs, including the cognate AHL signal for the LuxN domain, OHC4-HSL (Cao and Meighen 1989). In this study, we used double homologous recombination to develop the chimeric sensors LuxN_SphS and VanN_SphS, where the coding region of the native SphS gene was substituted by the chimeric genes, the expression of which were controlled by the native SphS promoter (Williams 1988; Shimura et al. 2012; Kotajima et al. 2014). The overexpression of HK and RR proteins *in vivo* may result in excess signal transduction (Krall and Reed 2000; Ehira and Ohmori 2006) and can be utilized to profile novel sensory kinases, as reported for the HK SasA, in the cyanobacterium *Synechococcus* sp. PCC 7942, that was identified by the mutation of EnvZ and PhoR in *E. coli* (Nagaya et al. 1993). Hence, the substitution of the coding region and the use of the native SphS promoter is important for such experiments. However, it has been previously reported that the expression levels of the native SphS protein are very low in the cells, and its expression is equally difficult to detect in the wild-type and in the transformants (Kimura et al. 2009; Inaba et al. 2018).

The other chimeric sensor, VanN_SphS, showed the most promising results in terms of suppression of AP activity by OHC6-HSL, which is the cognate AHL signaling molecule for the VanN sensor domain (Milton et al. 2001; Buchholtz et al. 2006). In the *Pseudomonas fluorescens* strain 2-79, among the six different AHLs (including C6-HSL and 3-OH-C8-HSL) tested in the extracts of the supernatant, the reporter strain (*phoA*::*lacZ*) could only respond to the OHC6-HSL (Khan et al. 2005). By the application of the gaseous stimuli, toluene, an increase in the induction of AP activity was observed until 32 h in the chimeric sensor TodSS_SphS in *Synechocystis* (Inaba et al. 2018). Contrary to this report, in our case, the addition of exogenous AHLs (OHC6-HSL) suppressed the AP activity compared to the control cultures of VanN_SphS. In a two-component transduction system, a short coiled-coil region serves as a connecter between the HK and RR domains and is known as the linker region. Thus, any changes in this region can affect the kinase signaling mechanism (Möglich et al. 2009; Bhate et al. 2015). Saita et al. (2015) observed that the stabilization/destabilization of the helical linker region of DesK, a thermo-sensory HK from *B. subtilis*, is important for regulating the signaling mechanism. Additionally, helix rotation and stretching were found to lead to an asymmetric kinase-competent state. Similarly, the blue-light-responding chimeric sensory

protein YFI showed distinct responses to light including variations in gene expression. Through the structural study of YFI, a rotation of 40°–60° was observed between the α-helix linker region and the HK domain under the light signal. This rotation activates the autophosphorylation of the His residue by regulating the interaction between the active site His residue in the HK domain and the adenosine triphosphate (ATP) binding site, resulting in a rotary-switch mechanism (Möglich et al. 2009).

In the same direction, Nakajima et al. (2016) developed a miniaturized CcaSs photoreceptor system by fusing the HK domains with a truncated linker region in the cyanobacterium *Synechocystis* sp. PCC6803 to construct various transformants. As a result, different response and gene induction mechanisms were observed in the developed transformants, which were grouped into four types. Type 1 showed gene expression induction under green light and suppression under red light, similar to the wild-type Ccas. Type 2 induced gene expression under both green and red light. However, type 3 showed a reverse gene induction mechanism from the wild-type, inducing gene expression by red light and its suppression by green light. Type 4 was unable to induce gene expression under any light. The authors proposed that the rotation in the angle of the α-helix linker region connected to the HK domain may result in the diverse responses to photo-regulation observed in these transformants. A similar structural effect may be present in our chimeric sensor VanN_SphS, inducing a base-line phosphorylation level that induces the expression of the *pho* regulon and could be also responsible for the absence of gene expression in the chimeric sensor LuxN_SphS. In all further experiments, only the chimeric sensor VanN_SphS was used, along with the AHL OHC6-HSL.

3.3. Effect of various concentrations of OHC6-HSL

To evaluate the effect of different concentrations of OHC6-HSL and to determine the minimal amount of this AHL needed to produce the response for VanN_SphS transformants, we measured AP activity after incubation with 0.1 μM, 1 μM, 2.5 μM, and 5 μM of OHC6-HSL. Here, 0 μM (no addition of OHC6-HSL) was used as a negative control, while 10 μM was used as the positive control. In this trial, different concentrations of OHC6-HSL were added to aerated culture tubes, and the changes in the AP activity were observed until 24 h. Among the tested concentrations, no concentration lower than 10 μM was found to effectively reduce the AP activity, showing a decrease in AP (48%) from 0.73 ± 0.008 to 0.38 ± 0.002 (μmol PNP mg^{-1}Chl*a* min^{-1}) within 0 to 24 h, respectively (Table 1). This result indicates that 10 μM is the only concentration of OHC6-HSL that may significantly activate our VanN_SphS transformants, and lower autoinducer concentrations are not as effective in suppressing AP

activity in our system. This amount of QS signal required to activate the chimeric sensor, despite being high, is within the range of AHL concentrations found in the culture medium of different bacteria, such as *Edwardsiella tarda* (Romero et al 2014).

3.4. Recovery of AP activity

In order to remove the OHC6-HSL after its addition to our system, we introduced an additional construct for the constitutive expression of Aii20J, a lactonase enzyme from the marine bacterium, *Tenacibaculum sp*. 20J, in *Synechocystis* cells harboring the VanN_SphS chimeric sensor and named it as VanN_SphS::Aii20J. It has been demonstrated that Aii20J degrades various unsubstituted (C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL) and substituted (OC6-HSL, OC10-HSL, OHC10-HSL, OC12-HSL, OHC12-HSL, OC13-HSL, and OC14-HSLOC6-HSL) AHLs (Mayer et al. 2015), making it suitable to degrade OHC6-HSL in our system, although it should be noted that the degradation activity is lower for substituted AHLs. In a first AP recovery experiment, we used Van_SphS::Aii20J cells, with VanN_SphS transformants as control cells. OHC6-HSL was applied at 10 µM in the aerated tubes of both strains, and AP activity was measured until 5.5 d. In this experiment, we observed a decrease in AP activity of both strains until 2 d after the addition of OHC6-HSL. Although the initiation of AP recovery was observed in the VanN_SphS::Aii20J cells, the rate of recovery remained low throughout the experiment and full recovery was not achieved until 5.5 d (Supplementary Fig. 2).

We hypothesized that light intensity may be a limiting factor for increasing cell density with time, which prevented VanN_SphS::Aii20J cells from fully recovering their AP activity. Asada et al. (2019) observed that for the induction of cell lysis in *Synechocystis*, along with phosphate deficient conditions, sufficient light irradiation in cultures is important. They demonstrated that inadequate light in dense cultures resulted in the suppression of AP activity. Adequate light supply promotes the regulation of gene expression in cyanobacteria (Mironov et al. 2012). To address this issue, we used a unique growth technique to improve AP activity, ensuring that the concentration of OHC6-HSL was not affected and cell density remained constant to an OD_{730} of 1.0 at 24-h intervals during the experiment (Supplementary Fig. 3). To maintain the cell density and OHC6-HSL concentration unaffected in the VanN_SphS and VanN_SphS::Aii20J cultures, a specific amount of cells, obtained using the formula mentioned in the materials and methods, was withdrawn from the aeration cultures of both strains, and after centrifugation, the supernatant was returned to the culture tubes (Supplementary Fig. 3 a, b). This allowed us

to maintain an optical density (OD_{730}) of 1.0 without changing the concentration of OHC6-HSL. The AP activity was measured from the cell pellet suspended in the new BG 11 media.

Initial AP activity at 0 d for VanN_SphS and VanN_SphS::Aii20J before the addition of OHC6-HSL was similar to the values of 0.67 ± 0.02 and 0.67 ± 0.05 µmol PNP mg^{-1}Chl*a* min^{-1}, respectively. The addition of 10 µM of OHC6-HSL resulted in a decline in AP activity in both strains until 2 d, reaching values of 0.09 ± 0.002 and 0.10 ± 0.023 (µmol PNP mg^{-1}Chl*a* min^{-1}) for VanN_SphS and VanN_SphS::Aii20J, respectively. Subsequently, the AP activity in VanN_SphS (control cells) remained constant during the experiment without showing any recovery in AP activity, with a value of 0.15 ± 0.01 (µmol PNP mg^{-1}Chl*a* min^{-1}) after 7 d of culturing. In the case of VanN_SphS::Aii20J cells, AP recovery was initiated after 2 d and increased with time as the administered OHC6-HSL was degraded by the Aii20J enzyme. A full recovery of AP activity was observed after 7 d, with a value of 0.69 ± 0.09 (µmol PNP mg^{-1}Chl*a* min^{-1}) (Fig. 3). Two-way ANOVA indicated a highly significant effect for the strain (VanN_SphS::Aii20J) ($p = 0.000$), time of application of OHC6-HSL (until 7 d) ($p = 0.000$), and their interaction (strain × time) ($p = 0.000$) compared to VanN_SphS (Fig. 3). Post-hoc analysis via Bonferroni correction also suggested a significant effect ($p = 0.009$) of VanN_SphS::Aii20J in quenching the OHC6-HSL and the recovery of AP activity compared to the control strain of VanN_SphS (Fig. 3).

To confirm the presence of AHL-degradation activity in the cultures, *C. violaceum* CV026-based plate bioassays in cultures of VanN_SphS and VanN_SphS::Aii20J were performed for 48 h. C6-HSL (10 µM) was used as degradation substrate instead of OHC6-HSL because the AHL-biosensor, *C. violaceum* CV026, does not respond to OHC6-HSL (Romero et al. 2010). We observed a gradual decrease in the concentration of C6-HSL in the supernatants of VanN_SphS compared to the control, which showed a reduction up to 30 % until 24 h. After 48 h, the HSL concentration in the VanN_SphS cultures became halved. In contrast, for the VanN_SphS::Aii20J cultures, the reduction in AHL concentration was higher, where in 24 h, 50 % reduction in the AHL concentration was observed and until 48 h, the AHL concentration (10 µM) was completely degraded by the QQ enzyme, Aii20J (data not shown). A very high specific activity was observed for the purified Aii20J enzyme to degrade C6-HSL and C10-HSL as compared to AiiA from *Bacillus* sp. However, the enzyme Aii20J lacks the presence of a signal peptide and the activity of the enzyme is believed to be mainly intracellular. Therefore, the exogenous application of the enzyme may result in a higher activity to degrade the specific AHLs (Romero et al. 2014; Mayer et al. 2015).

In several studies, the effect of various other AHL lactonases on the degradation of different AHLs has been demonstrated. AHL lactonase Aii810 degrades *N*-butyryl-L-homoserine lactone and *N*-3-oxododecanoyl-L-homoserine lactone in *P. aeruginosa* (Fan et al. 2017), the degradation of multiple AHLs by HqiA has been

observed in plant-pathogen *Pectobacterium carotovorum* (Torres et al. 2017), and MomL from *Muricauda olearia* Th120 showed effective degradation of both short- and long-chain AHLs, including C6-HSL (Tang et al. 2015).

3.5. Expression of phoA *mRNA through real-time PCR*

The suppression of AP activity in VanN_SphS cells by the addition of OHC6-HSL and its role as a negative regulator of the AP assay was further confirmed by measuring the relative expression of the *phoA* mRNA genes. Real-time PCR was used to determine the relative expression of *phoA* mRNA with and without the application of OHC6-HSL (10 μM) in VanN_SphS cells. The samples were taken every 10 min for 60 min. The expression of -10 min culture cells was used as the base for calibration. As expected, the addition of OHC6-HSL resulted in a decrease in *pho*A gene expression over time compared to the VanN_SphS cultures not exposed to OHC6-HSL. The relative expression of *phoA* mRNA at 60 min as a control of VanN_SphS cultures (-OHC6-HSL) was 1.27 ± 0.28, and in VanN_SphS cultures (+OHC6-HSL) was 0.14 ± 0.15, with a decrease of 92 % (Fig. 4). Two-way ANOVA (time × AHL) (-10 to 60 min) revealed a highly significant ($p = 0.0004$) effect of time and OHC6-HSL application ($p = 0.0002$) on the VanN_SphS cells exposed to OHC6-HSL in decreasing *pho*A mRNA expression, the interaction of which (time × AHL) also had a highly significant effect ($p = 0.0038$). Bonferroni correction showed that the application of OHC6-HSL in VanN_SphS cells significantly decreased ($p = 0.004$) *phoA* mRNA expression within 60 min compared to the VanN_SphS cells without OHC6-HSL (Fig. 4). Inaba et al. (2018) observed an increase (15 times higher) in the relative *phoA* mRNA expression in the cells of the chimeric sensor TodSS_SphS when exposed to the exogenously applied gaseous compound, toluene, within 5 h of exposure compared to the control cells. Our results confirmed that exogenously applied OHC6-HSL inhibited the expression of *phoA* mRNA compared to the cultures not exposed to this AHL, indicating the successful regulation of gene expression.

4. Conclusion

This study is the first report on the utilization of QS molecules (AHLs) and QS domains to develop a chimeric sensor system in *Synechocystis* for the regulation of gene expression. We successfully developed a gene switch OFF/ON system for the regulation of AP activity in *Synechocystis*, where the application of an exogenous compound, AHL (OHC6-HSL), can suppress AP activity in the chimeric sensor VanN_SphS (gene switch OFF),

whereas, by the expression of the QQ enzyme, Aii20J, the administrated AHL was gradually degraded and AP recovered to its original level (gene switch ON). Our results may be useful in future studies for the development of artificial gene regulatory systems in cyanobacteria.

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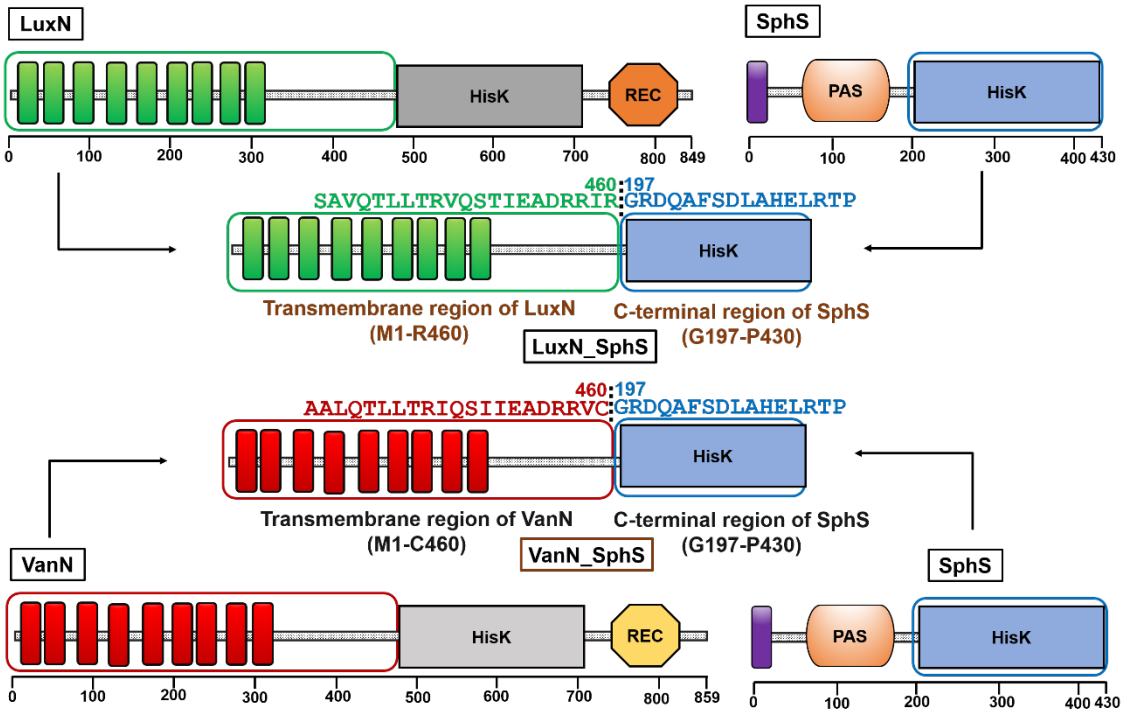

Fig. 1 A schematic depiction of the chimeric sensors LuxN_SphS and VanN_SphS. Illustration of sensor domain, LuxN, from *Vibrio harveyi* BB120 and its homolog, VanN, from *Vibrio anguillarum* 90-11-287 along with histidine kinase, SphS (sll0337), a Pi-deficient sensory kinase domain from cyanobacterium *Synechocystis* sp. PCC 6803. LuxN_SphS chimeric sensor represents the fusion of the transmembrane region (green rectangle) of sensory domain LuxN (M1-R460) and C-terminal region (blue rectangle) of SphS (G197-P430). VanN transmembrane region (red rectangle) (M1-C460) was fused with the C-terminal region (blue rectangle) of SphS (G197-P430) to obtain chimeric protein VanN_SphS. The N-terminal of SphS comprises, a short transmembrane region (hydrophobic) and PAS (Per-Arnt-Sim) domain, responsible to regulate the redox potential, oxygen, small ligands, and cellular energy level. Histidine kinase (HisK) causes the autophosphorylation of a specific signal and transfers the phosphoryl group to the Receiver (Rec) domain, causing a specific response to the original signal. The scale shows the positions of amino acids in each domain.

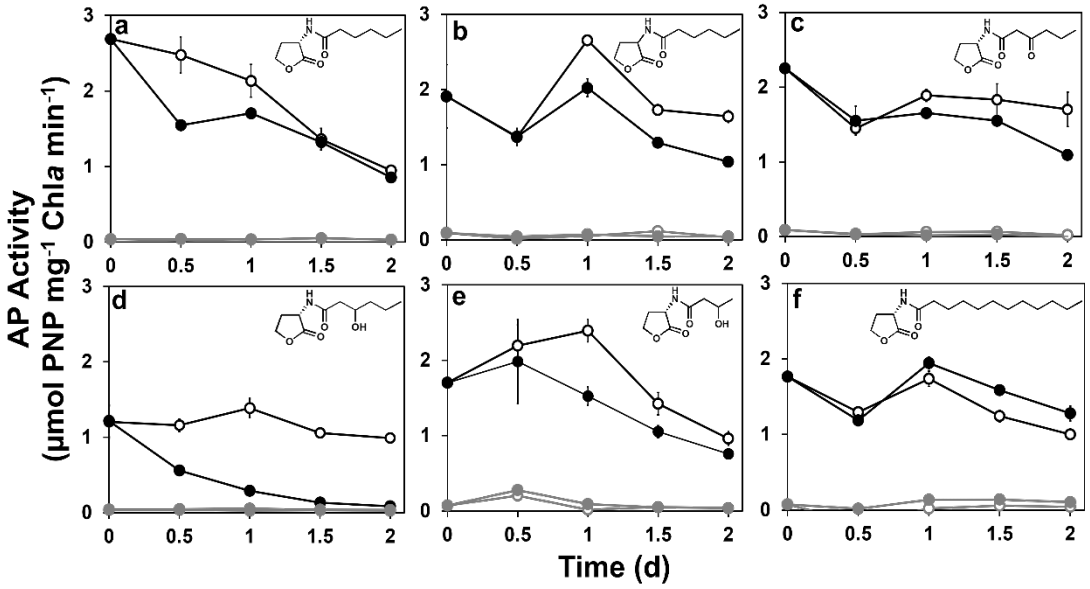

Fig. 2 Activity of the alkaline phosphatase (AP) in the chimeric sensors LuxN_SphS cells (gray lines) and VanN_SphS cells (black lines) with (close circles) and without (open circles) addition of various chain length *N*-acyl-homoserines lactones (AHLs) for 2 d. All AHLs were applied at a final concentration of 10 μM in the cultures of LuxN_SphS and VanN_SphS (a). Effect of *N*-hexanoyl-L-homoserine lactone (C6-HSL) (b). *N*-hexanoyl-DL-homoserine lactone (C6-HSL racemic) (c). *N*-3-hydroxybutyryl-L-homoserine lactone (OHC4-HSL) (d). *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) (e). *N*-(*β*-ketocaproyl)-L-homoserine lactone (OC6-HSL) (f). *N*-dodecanoyl-L-homoserine lactone (C12-HSL). Data represent the mean ± standard deviation (SD) of three independent biological replicates.

Fig. 3

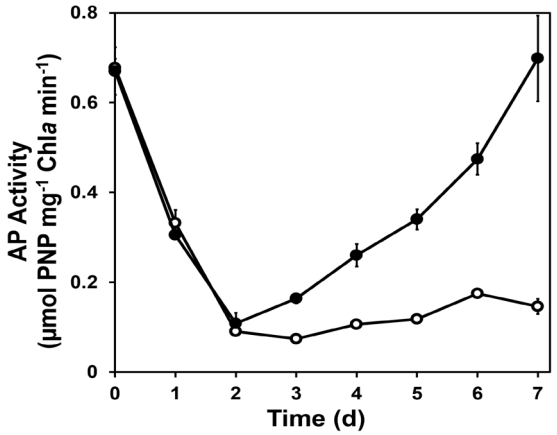

Fig. 3 Recovery of alkaline phosphatase activity (AP) by the degradation of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) via quorum quenching enzyme, Aii20J, under special growth conditions. Alkaline phosphatase (AP) activity in the control strain VanN_SphS cells (open circles) and VanN_SphS::Aii20J strain (filled circles) with the addition of 10 μM *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) for 7 d. Data represent the mean ± standard deviation (SD) of three independent biological replicates.

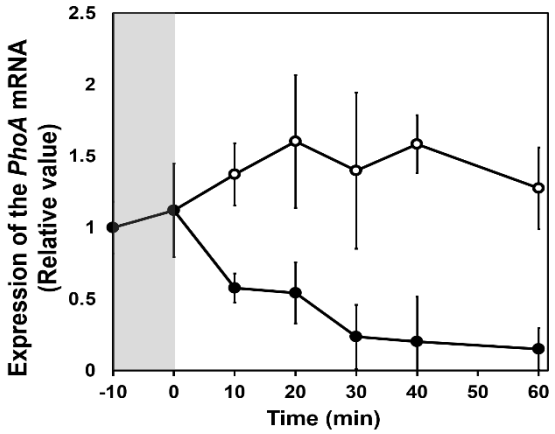

Fig. 4 Relative *phoA* mRNA expression in VanN_SphS cells with and without the addition of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) for 60 min. The shaded part indicates the time before the addition of OHC6-HSL. The open and close circles represent VanN_SphS cells without and with the addition of OHC6-HSL, respectively. Data represent the mean ± standard deviation (SD) of three independent biological replicates.

Table 1 Effect of different concentrations (0.1, 1, 2.5, 5, and 10 μM) of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) on the alkaline phosphatase (AP) activity (μmol PNP mg^{-1}Chl*a* min^{-1}) of chimeric sensor VanN_SphS cells until 24 h. Data represent the mean ± standard deviation (SD) of three independent biological replicates.

Concentration of OHC6-HSL (μM)	Time		% decrease in AP activity*
	0 h	24 h	
0	0.95 ± 0.048	1.20 ± 0.103	-26.3
0.1	1.03 ± 0.016	1.19 ± 0.008	-15.5
1	0.99 ± 0.031	0.92 ± 0.030	7.1
2.5	0.75 ± 0.014	0.89 ± 0.037	-18.7
5	1.68 ± 0.047	1.29 ± 0.034	23.2
10	0.73 ± 0.008	0.38 ± 0.002	47.9

*(0 h - 24 h) × 100 / 0 h

Supplementary Table 1 Primers used for the construction of LuxN_SphS, VanN_SphS, and VanN_SphS::Aii20J strains and the measurement of *Pho*A gene expression using real-time PCR.

Primers	Sequences (5' to 3')
Chimeric sensors LuxN_SphS and VanN_SphS	
LuxN_F	GGCAACTGCTAGAACATGTTTGATTTTAGCCTAGA
LuxN_R	AGCTTGATCCCTACCTCGAATACGGCGATCCGCTT
VanN_F	GGCAACTGCTAGAACATGCTTAACCTCAACTTAGA
VanN_R	AGCTTGATCCCTACCTCGAATACGGCGATCCGCTT
For AHL-quenching strain VanN_SphS::Aii20J	
βLAC_F	ACGATAAGGATCATACATATGAAAAAGATTTTCTTACTAG
βLAC_R	TGAGGTTAACAGATCAGATCTTATTTCTTCAAGAGATTC
pTCHT_srl2031_F	AGATCTGTTAACCTCACATTGG
pTCHT_srl2031_R	TATGATCCTTATCGTCATCGTC
For Real-time PCR	
PhoA_RT_F	CAGTGGCTTTGCCTTCAGTTT
PhoA_RT_R	ACCATTACGCACAACAACATCC
rnpB_RT_F	GTAAGAGCGCACCAGCAGTATC
rnpB_RT_R	TCAAGCGGTTCCACCAATC

Aii20J ->

100

ATGAAAAAAATATTTTTATTAGCTCTTACGACTATTATTACATTTAGTTGTAAAAATGCCGAAAAGAAGCAAACAACAGAAGAAAAAACAG
TTGAAAAGC

M K K I F L L A L T T I I T F S C K N A E K K Q T T E E K T V E K P

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CGTTGAGAAAC

200

CTCAAGTAAAACTTCATGTTTTAGATGGAGGTTCAATTTTAGTTAACAAACTTGAAGTTTTTTCTCAAGATACAACATACACAGGACAGT
CTAAACAGTT

Q V K L H V L D G G S I L V N K L E V F S Q D T T Y T G Q S K Q F

CCCAAGTGAAACTGCACGTGTTAGATGGTGGCTCTATCTTGGTGAATAAACTCGAAGTGTTTTCTCAAGACACAACCTATACCGGTCA
GAGCAAACAGTT

300

TTCAGATGCTTACTATGTAATATCTCACCCTAAAGGAAATTTAATGTGGGATGCTGGTTTACCTGAAGCACTAATTACTGACGAACCTTTT
ACAGAGCCT

S D A Y Y V I S H P K G N L M W D A G L P E A L I T D E P F T E P

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400

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TCTCATC

S G T F T L Q R K D S L K N Q L K S I G L T V D D F K Y F V L S H P

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CTTAAGTCATC

500

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CTGCAAAAGT

H F D H T G H A N Y L K N A T W L V Q E N E Y N F I T N D S A K V

CCCATTTTGACCATACTGGACATGCGAACTATCTGAAAAATGCCACATGGTTAGTTCAAGAGAACGAGTATAACTTCATTACCAATGATT
CAGCCAAAGT

600

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K D P D T Y N S I K E L K N V E K I N G D H D V F G D G T V V I K

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Y M P G H T I G H Q A L Y I E A G L E K P I L L T G D L Y H F E E N

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800

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R E T K G V P S F N Y D V E Q T L E S M K K F E A F A K E K N A E

ATCGCGAAACAAAAGGGGTACCTAGCTTTAACTATGATGTCGAACAAACGTTGGAAAGCATGAAGAAGTTTGAAGCATTTGCCAAAGA
AAAAAACGCTGA

861

GGTGATTATTCAACACTCACCAAAAGATTTCAAAAAATTACAAAATCTATTAAAAAAGTAA

V I I Q H S P K D F K K L Q N L L K K *

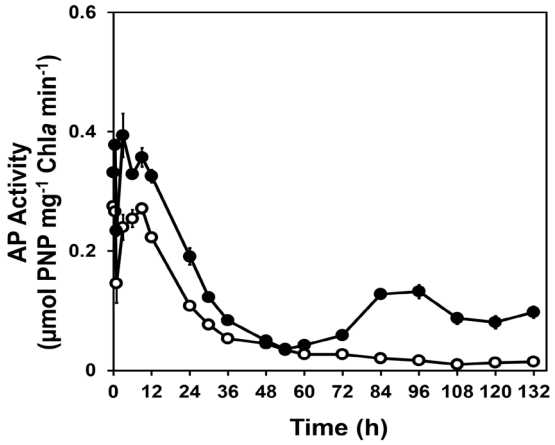
AGTGATTATTCAGCATAGTCCCAAGGACTTCAAGAAACTGCAGAATCTCTTGAAGAAATAA

Supplementary Fig. 1 The nucleotide sequence of the lactonase enzyme, Aii20J. The upper lines represent the original sequence, while lower lines indicate the codon-optimized sequence of Aii20J. The modified nucleotides are shown as red characters. The numbers represent the nucleotide position from the 5’ end.

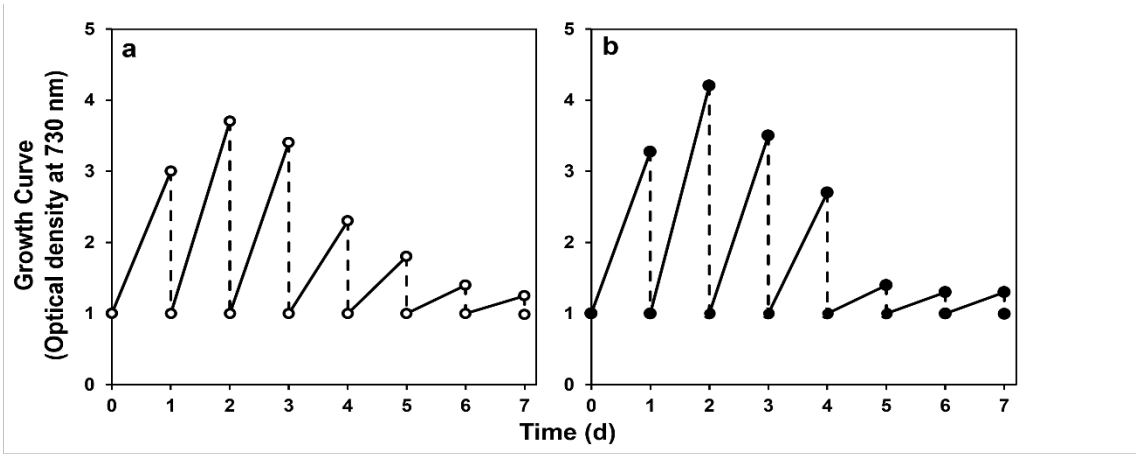
1

2

3



Supplementary Fig. 2 Recovery of alkaline phosphatase (AP) by the degradation of *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) via quorum quenching enzyme, Aii20J, using continuous growth cultures. AP activity in the control strain VanN_SphS cells (open circles) and VanN_SphS::Aii20J strain (filled circles) with the addition of 10 μM *N*-3-hydroxyhexanoyl-L-homoserine lactone (OHC6-HSL) up to 132 h under normal continuous growth conditions. Data represent the mean ± standard deviation (SD) of three independent biological replicates.



Supplementary Fig. 3 Special growth technique for the recovery of alkaline phosphatase activity by the dilution of cultures and maintaining the cell optical density constant (OD_{730} = 1) throughout the experiment. (a) Changes in the growth of VanN_SphS cultures (Solid line) and diluting the cells (dotted line) to maintain the cell density to OD_{730} = 1 within 7 d. (b) Changes in the growth of VanN_SphS::Aii20J cultures (solid line) and diluting the cells (dotted line) to maintain the cell density to OD_{730} = 1 within 7 d.