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ORIGINAL ARTICLE

Control of virulence factor expression in opportunistic pathogens using cyclodextrin immobilized gel

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Abstract Cyclodextrins (CDs) could intercept a quorum sensing (QS) in *Pseudomonas aeruginosa* by trapping signal molecules for bacterial cell-to-cell communication systems. The QS in human pathogens is the cell density dependent system that regulates the expression of virulence determinants. The cellulose ether hydrogel immobilized with 2-hydroxypropyl-β-CD (HP-β-CD) was synthesized as an artificial QS signal receptor to develop a novel system for controlling the expression of virulence determinants in *P. aeruginosa*. Since the α- and β-CD could form inclusion complex with *N*-acyl-L-homoserinelactone (AHL) as the gram-negative bacterial QS signal in the culture solution, both AHL-mediated *rhl* and *las* quorum-sensing systems could be intercepted by CD. Inhibitory control of the *rhlA* transcription could cause to produce less pyocyanin in the presence of 5 mM soluble α- or β-CD in the liquid medium. Furthermore, production of the reporter enzyme β-galactosidase was meaningfully decreased in *P. aeruginosa* PAO1 (pQF50-*lasB*) containing *lasB-lacZ* transcriptional fusion when HP-β-CD immobilized gel was immersed in the culture solution to trap AHLs from the liquid medium. By adding the artificial AHL receptors to the culture medium, the expression of the QS-controlled genes could be remotely controlled from the outside of cells.

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Introduction

Antibiotic resistance of bacteria is becoming a serious problem. Since bacteria are single-celled creatures, there is a possibility that some infectious bacteria adapt quickly to new environmental conditions due to over-use of antibiotics. It is necessary to develop a preventive material for the infectious disease avoiding antibiotic use. To solve the problem, a novel system will be proposed as the artificial control of cell-to-cell communication systems in opportunistic human pathogens.

Communication systems in bacteria have been recently reported in both gram-positive and gram-negative bacteria [1]. Among them, more attention has given to controlling quorum sensing (QS) system in gram-negative bacteria because they play an important role for the infectious disease control in human pathogens. The QS system is one of the cell-to-cell communication mechanisms and relied on the signal concentration, because the signal molecules are considered to diffuse inside and outside of cells. Therefore, signal accumulation due to an increase of the cell population density is an essential key for the expression of the QS-dependent genes.

Our proposing system is to artificially reduce the QS signal concentration by trapping signals onto polymer gel sheets immobilized with an artificial receptor for the QS signal (Fig. 1). In our previous paper, some kinds of cyclodextrins (CDs) were

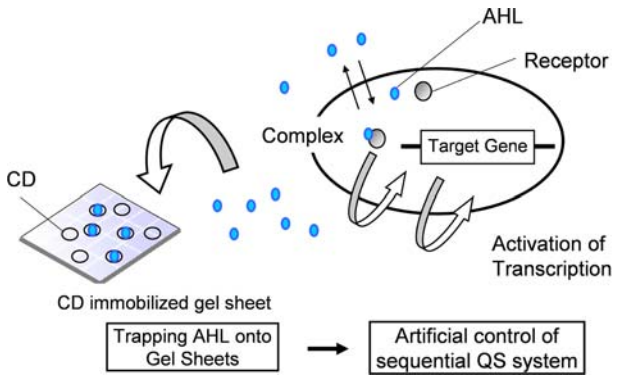

Fig. 1 Control of quorum sensing with artificial AHL receptors immobilized on hydrogel sheets. Trapping AHL signals onto the gel sheets can intercept the sequential QS system due to decreasing the local AHL concentration

reported to effectively operate as the artificial receptors for common QS signal, *N*-acyl-L-homoserine lactones (AHLs) that are produced in gram-negative bacteria. CD immobilized polymer gel sheets could control the AHL-mediated QS system in *Serratia* species [2]. The effects of the CD immobilized gel sheets were investigated for controlling the prodigiosin production that was regulated by AHL-mediated QS system. The bacterial QS is the cell density-dependent mechanism that triggers to activate transcription of targeted genes. Briefly, the complex between the AHL and its receptor protein becomes stable in cells when the local AHL concentration reaches the threshold. Accordingly, the sequential QS system will be activated by increasing cell population density; the QS system regulates respective functions including virulence factor expression, biofilm formation, bioluminescence, etc.

In this report, the hydrogel immobilized with artificial AHL receptors would be applied to the QS control in *Pseudomonas aeruginosa.* The expression of multiple virulence genes relied on the QS system in *P. aeruginosa* that occasionally causes serious infectious disease.

Experimental

Materials

2-Hydroxypropyl cellulose (HPC, Mw 100, 000) and 2-hydroxypropyl-β-CD (HP-β-CD) were purchased from Acros Organics. Divinyl sulfone (DVS) was purchased from Wako Pure Chemical Industries. All other chemicals were of reagent grade.

Bacterial strain and culture condition

The two AHL signaling systems are *rhlA* and *lasB* that respectively code rahmnosyltransferase and elastase in *P. aeruginosa* (Fig. 2a). The AHL complex with each receptor protein can activate transcription of respective genes. *P. aeruginosa* AS-3 was grown at 30 °C for 18 h in Luria-Bertani (LB) medium. To study the CD effects on activation of *rhl* quorum-sensing system, a change in pyocyanin production was analyzed. Pyocyanin is the well-studied blue-green phenazine pigment that was known as one of secondary metabolites through the *rhl* quorum-sensing system [3].

To investigate the regulation of the *las* quorum-sensing system, *P. aeruginosa* PAO1 (pQF50-*lasB*) was grown under the same condition of *P. aeruginosa* AS-3. This reporter strain possesses a pQF50 derivative containing the *lasB-lacZ* fusion, where the *lacZ* gene codes the β-galactosidase [4]. As shown in Fig. 2b, activity of the reporter enzyme was determined as the

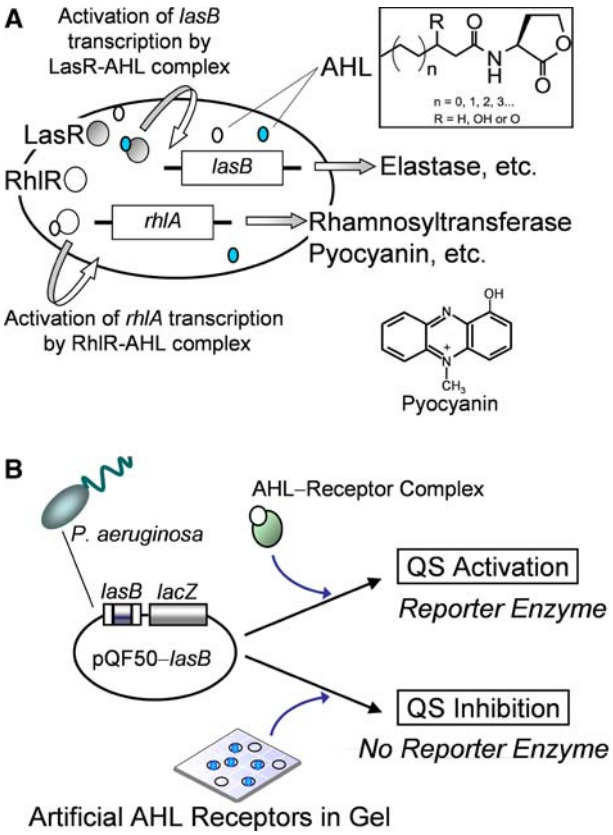

Fig. 2 (**a**) The *rhl* and *las* quorum-sensing systems in *P. aeruginosa*. Both AHL-mediated circuitries operate in a hierarchical cascade responsible for controlling the virulence factor expressions and the secondary metabolites. (**b**) Evaluation of the *lasB* expression by *lasB-lacZ* transcriptional fusion

index of artificial control of the *las* quorum-sensing system.

Preparation of gel sheets

A basic preparation procedure of cellulose ether gel was described in the previous paper [5]. Desired amount of DVS was added to pre-gel solution containing 3.5 wt% HPC and 3.5 wt% HP-*β*-CD. After stirring for 30 s, the mixture was poured into the glass mold separated by silicone rubber gasket (1.5 mm thick). The CD was immobilized onto the polymer network at the same time of the crosslinking of HPC polymers. The prepared gel sheets were cut into pieces of 5 × 5 × 1.5 mm in size just after separation from the glass mold. Immobilized CD amount was calculated by the amount of expelled CD into the washed solution after immersing the gel sheets in diluted HCl solution and distilled water. By refractive index measurement (Shimadzu RID-6A), it appeared that more than 95% CD in the pre-gel solution was immobilized on the gel sheets. For the preparation of conventional HPC gel sheets without CDs, DVS was added to 7.0 wt% HPC aqueous solution.

Pyocyanin assay

After the cell growth of *P. aeruginosa* AS-3, the color of the culture solution turned into green due to the pyocyanin production. Pyocyanin was extracted from the desired amount of culture solution to chloroform and then re-extracted into diluted HCl solution to determine its amount by measuring the absorbance at 520 nm (A_{520}), because the color of the pyocyanin turned into red under acidic condition. The pyocyanin production was calculated as per cells, of which density was determined as the turbidity of the culture solution (OD_{600}). To study the soluble CD effects onto the pyocyanin production, *P. aeruginosa* AS-3 was grown in the presence of 5 mM α-, *β*-, or *γ*-CD. The pyocyanin production was expressed as the relative value after the normalization by the control without CDs.

β-Galactosidase assay

The reporter enzyme *β*-galactosidase was extracted from the culture solution of *P. aeruginosa* PAO1 (pQF50-*lasB*). After dilution of the culture solution in the phosphate buffer solution (pH7.0), the cells were disrupted and then collected by centrifugation. *β*-Galactosidase activity in the supernatant was measured after mixing the 2.17 mM *o*-nitrophenyl-*β*-galactopyranoside (ONPG) aqueous solution that was filled in a thermoelectric peltier cell of the UV-VIS spectrophotometer (JASCO International Co. V-550). Time evolution of the absorbance at 420 nm (A_{420}) was determined after adding the supernatant to the ONPG solution at 37 °C. Since the A_{420} linearly increased with time, the *β*-galactosidase activity was determined from the slope of the straight line. The relative *β*-galactosidase activity was calculated as per cells using the turbidity of the culture solution (OD_{600}). The *β*-galactosidase production was normalized with that of the control without gel sheets. Immersing the HP-*β*-CD immobilized gel sheets during the cell growth could drastically decrease the *β*-galactosidase activity.

Results and discussion

Control of the *rhl* quorum-sensing system with CDs

The *rhl* and *las* quorum-sensing circuitries operate in a hierarchical cascade involving their signal molecules *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL). In our previous papers, it was reported that acyl chains of the AHLs could be included into the hydrophobic cavity of CDs in aqueous solution [2, 6]. The QS in *Serratia* Sp. could be inhibitory controlled in the presence of soluble or immobilized CDs in the culture medium. In this research, this proposing system would be applied to the control of *rhl* and *las* systems in *P. aeruginosa*.

In Fig. 3, the relative pyocyanin production in *P. aeruginosa* AS-3 was shown in the presence of

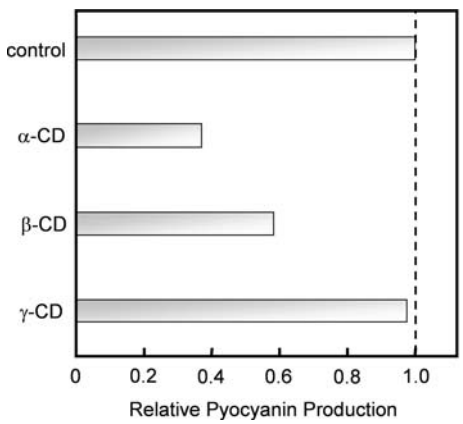

Fig. 3 Control of the relative pyocyanin production with addition of soluble cyclodextrins. *P. aeruginosa* AS-3 was grown in 4 ml of the LB medium in the presence of 5 mM CDs. The pyocyanin production was determined as per cells after extraction in diluted HCl solution

soluble CDs with differently sized cavities. The pyocyanin production was defined as the ratio of the absorbance of the extracted pyocyanin solution at 520 nm to turbidity of the culture suspension at OD_{600}. The cell growth was not affected by adding any CDs in the liquid medium. The relative pyocyanin production was meaningfully reduced to approximately 0.4 in the presence of 5 mM α-CD. Since the effect depends upon the cavity size of the CDs, inclusion complex formation between AHLs and CDs plays an important role for controlling the AHL-mediated pyocyanin production. Transcriptional control of *rhlA* gene is probably responsible for decreasing pyocyanin, of which production can be activated by increasing the AHL concentration above the threshold.

Control of the *las* quorum-sensing system with CDs

To immobilize the CD molecules onto the three-dimensional polymer network, HPC was selected as the polymer backbone because both CDs and cellulose derivatives possess the same glucopyranose units. It was reported that DVS could quantitatively react to crosslink between two hydroxy groups under alkaline condition. HP-β-CD immobilized HPC gel was designed as the 3D-network possessing the same 2-hydroxypropyl side chains. A gel sheet (5 × 5 × 1.5 mm) containing approximately 0.85 μmol of HP-β-CD was immersed in 4 ml of liquid medium and then 1% of *P. aeruginosa* PAO1 (pQF50-*lasB*) preculture was inoculated.The effects of the CD immobilized gel sheets were studied through the expression of a *lasB-lacZ* transcriptional fusion, because AHL capture onto the gel sheets can effectively decrease the local AHL concentration and intercept the hierarchical QS system. After 18-h shaking, β-galactosidase activity was measured by the modified Miller's procedure [7].

Figure 4 shows the time evolution of the absorbance at 420 nm as *o*-nitrophenol concentration derived from the hydrolysis of the substrate ONPG. The enzymatic activity was determined in pH 7 buffer solution containing 2.17 mM ONPG at 37 °C. The activity obviously became small when four CD immobilized gel sheets (5 × 5 × 1.5 mm) were immersed in the culture solution during the cell growth. Since both plots with or without CD immobilized gel sheets fell on straight lines, the enzymatic activity was calculated from the slopes (m's) of the straight lines. The relative β-galactosidase activity was calculated from A_{420}/OD_{600} and summarized in Table 1.

The cell density after 18-h culture was not affected by immersion of the gel sheets. The relative β-galactosidase activity had a positive correlation with the transcriptional activity of the *lasB* gene. As compared with the control value, conventional HPC gel sheets had small effects to decrease the relative β-galactosidase activity because the gel sheets did not contain any CDs. This result indicated that nonspecific AHL adsorption onto the polymer network was small to affect the sequential QS process. A similar result was already obtained for the QS control in *Serratia* sp. as one of opportunistic human pathogens [2]. Values of the relative β-galactosidase activity decreased with increasing the number of gel sheets and reached approximately 0.36 when eight gel sheets were immersed in four ml of the culture solution during the cell growth. The result shows that the AHL molecules could be trapped by CD even after immobilization onto the cellulose ether gel.

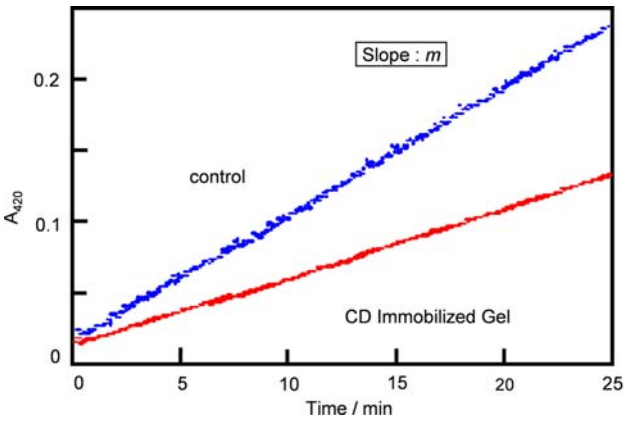

Fig. 4 Determination of the β-galactosidase activity with the modified Miller's method

Conclusion

The QS systems in *P. aeruginosa* could be effectively controlled by soluble or immobilized CDs. Since the expression of both *rhl* and *las* quorum-sensing systems

Table 1 Control of the relative β-galactosidase activity with CD-immobilized gel sheets

		Relative β-galactosidase activity[a]
Control		1.00
HPC gel[b]	4 sheets	0.95
HPC/HP-β-CD gel[b]	1 sheet	0.69
	4 sheets	0.57
	8 sheets	0.36

[a] *P. aeruginosa* PAO1 (pQF50-*lasB*) containing the *lasB-lacZ* transcriptional fusion

[b] Desired number of gel sheets (5 × 5 × 1.5 mm) were immersed in 4 ml of the LB medium

were intercepted, the AHL capture onto the CD is probably responsible for decreasing the AHL signal concentration in cells. Since artificial control of virulence factor expression in *P. aeruginosa* is seriously needed to prevent infectious disease, a novel strategy will solve the problem on the antibiotic resistance of infectious bacteria. Natural oligosaccharides cyclodextrins are safe materials for human. The CD immobilized cellulose hydrogel is one of the promising materials for the artificial receptor to regulate the QS-dependent gene transcription.

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