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Regulation of Quorum Sensing in *Serratia marcescens* by Adsorption of *N*-Acylhomeserine Lactones on Cyclodextrin Immobilized Gel

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Inhibitory control of quorum sensing (QS) was realized using the cyclodextrin (CD) immobilized hydrogels that function as an artificial receptor to gram-negative bacterial signal molecules. QS is a mechanism for controlling gene expression in response to cell population density. *Serratia marcescens* regulates red pigment (prodigiosin) production with the QS system mediated by *N*-acylhomoserine lactone (AHL); AHLs are common signal molecules for various gram-negative bacteria. Prodigiosin production could be effectively inhibited when the CD gel sheets were immersed in the culture medium during the cell growth. Inclusion complex formation is responsible for decrement of AHL concentration inside and outside of cells and inhibitory control of transcription for QS-regulated genes. To elucidate the CD-AHL inclusion complex formation for the differently sized CD cavity, α-CD or γ-CD was immobilized on cellulose ether hydrogels. After *S. marcescens* was cultured in the presence or absence of CD immobilized gel sheet in liquid medium, intracellular prodigiosin was extracted with aqueous ethanol solution and determined by absorbance measurement. Immobilized α-CD on hydroxypropyl cellulose gel sheets decreased prodigiosin production up to approximately 50% at 30°C and 26% at 25°C. These results indicated that CD gels have high potential of controlling QS by trapping bacterium signal compounds for cell-cell communication.
Key words: quorum sensing, cell-cell communication, cyclodextrin, polymer gel, bacterial signal molecule

1. INTRODUCTION

Hydrogel is a three-dimensional polymer network swollen in aqueous milieu. Soft and wet properties of hydrogels are expected to use with great potential for medical and biological applications such as biomimetic materials, biocompatible materials, drug carriers, etc. In this report, a novel system for controlling bacterial cell-to-cell communication is proposed using cyclodextrin (CD) immobilized hydrogels as artificial receptors of bacterial signal molecules.

Cell-to-cell communication by means of small signal molecules plays important roles for adaptation of bacteria to the surroundings. Quorum sensing (QS) is the mechanism that allows bacteria to sense the density of the surrounding bacterial population and to respond to the information for controlling some gene expression [1,2]. At low cell population densities, concentration of diffusible signal molecules, known as autoinducer (AI), remained low. In the event of increasing cell densities, local AI concentration increases and then reaches a threshold value. Under the condition, a complex between receptor protein and AI becomes stable in cells and begins to activate transcription of the QS-regulated genes. It is known that gram-negative bacteria produce acylated homoserine lactones (AHLs) as signal molecules, while AI of gram-positive bacteria is specific small peptides [1].

To artificially control the QS system, some methods were proposed, including enzymatic degradation of AHL [3] and competitive inhibition with AHL antagonists [4-6]. Furthermore, AHL capture was expected to effectively regulate the QS system; we previously reported that CDs easily form inclusion complexes with AHL molecules. ROESY spectrum clearly showed that the hydrophobic cavity of α- and β-CD can interact with acyl chains of AHLs [7]. Consequently, CD molecules were immobilized on hydrogel sheets to create a novel AHL trapping system in the bacterial culture medium.

Serratia marcescens is classified into a gram-negative bacteria, of which characteristic feature is to produce the intracellular red pigment, 2-methyl-3-pentyl-6-methoxy prodigiosin [8, 9]. This prodigiosin production is controlled via AHL mediated QS system [10]. Artificial reduction of AHL concentration is expected to lead inhibitory control of prodigiosin production.

The purpose of this research is to artificially control the AHL-mediated QS system of gram-negative bacteria by using CD hydrogel sheets. Effects of CDs and CD-immobilized hydrogels are elucidated in respect of prodigiosin production of *S. marcescens*.

2. EXPERIMENTAL

2.1 Materials

α-, β-, and γ-CD were commercial product of Wako Pure Chemical. Hydroxypropyl cellulose (HPC) and hydroxyethyl cellulose (HEC), of which average molecular weights respectively were 100,000 and 90,000, were purchased from Acros. Hydroxypropyl methyl cellulose (HPMC) was a commercial product as Methocel-E (average molecular weight: 10,000) purchased from Dow Chemical. All other chemicals were of reagent grade.

2.2 Bacterial strain and culture conditions

S. marcescens isolated from an aeration tank of activated sludge (Utsunomiya, Japan) was grown at 30 or 25°C in Luria-Bertani (LB) medium with or without 5

mM α-, β-, or γ-CD. To elucidate the effects of hydrogel sheets on prodigiosin production, five gel sheets (10 × 10 × 1.5 mm^3) were immersed in 4 m*l* of liquid medium and then 1% of *S. marcescens* preculture was inoculated. Approximately 80 μmol of α-CD was immobilized within the gel sheets that were added in 4 m*l* of LB medium.

2.3 Gel synthesis

A basic scheme of cellulose ether gel preparation [11] was modified to synthesize the CD immobilized gel sheets. Dry HPC powder (3.5 wt%) and α-CD (3.5 wt%) were dissolved in NaOH solution (pH 12). After addition of 3.0 wt% divinyl sulfone (DVS), the solution was stirred thoroughly for 30 s and poured between two glass plates separated with silicone rubber gasket (1.5 mm thick). The reaction was carried out at 22°C for 24h. Similarly, HPC gel sheets immobilized with γ-CD were synthesized as the same manner. The gel sheet separated from the mold was fully washed with diluted HCl solution and water. Any non-immobilized CDs diffused out to the washed solution were analyzed by HPLC equipped with refractive index detector (Shimadzu RID-6A). More than 95% CD molecules in the pre-gel solution were immobilized on gel sheets.

2.4 Dynamic mechanical measurements

The compressive modulus, $E^* = E' + iE''$, was obtained by thermally mechanical analyzer (Seiko Instrument Co. TMA/SS6100), where E', E'', and tanδ are the storage modulus, loss modulus, and loss angle ($\tan\delta = E''/E'$), respectively. A synthesized wave stress (0.1 Hz) was loaded on the gel sheet with measuring the wave strain, while the strain was kept within 1% of the gel thickness. Any measurements were brought about with the equilibrated gel sheets at 22°C in water.

2.5 Assay of prodigiosin production

The relative prodigiosin production was quantified as follows [12]. One m*l* of cultured medium was centrifuged at 13,200 rpm for 5 min. After separation of the supernatant to harvest cells and remove any CD molecules dissolved in the medium, the pellet re-suspended in 1 m*l* of acidified ethanol (2% 2M HCl in ethanol) to extract the prodigiosin from cells. The mixture was centrifuged again to remove cell debris and the absorbance at 534 nm (A_{534}) of the supernatant was determined by UV-VIS spectrophotometer (JASCO V-550DS). Prodigiosin production was calculated per cell as the ratio of A_{534} and OD_{600}. Effects of CDs or CD immobilized gel sheets were evaluated as the relative prodigiosin production, where the control value of A_{534}/OD_{600} equals to one.

3. RESULTS AND DISCUSSION

3.1 Inhibitory effects of CD on prodigiosin production

It was reported that *S. marcescens* produces at least four classes of AHLs as *N*-(3-oxohexanoyl)-L- (3-oxo-C6-HSL), *N*-hexanoyl-L- (C6-HSL), *N*-heptanoyl-L- (C7-HSL), and *N*-octanoyl-L-homoserine lactone (C8-HSL) [10]. However, no report appeared concerning details of each AHL role for *S. marcescens* in respect of quorum sensing. In our previous report, inclusion complex formation between CD molecules and AHLs

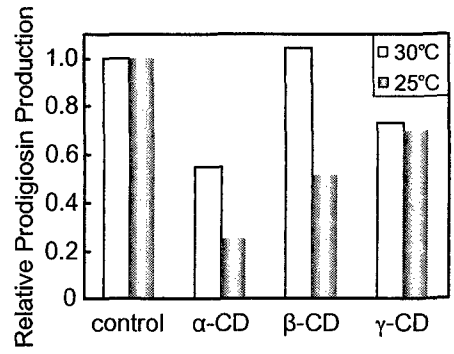

Fig. 1 Effects of cyclodextrin molecules on relative prodigiosin production in *S. marcescens*. *S. marcescens* was grown at 30 or 25°C in the presence of 5 mM CD for 24 h.

was investigated by 1D ^{1}H-NMR and ROESY experiments using chemically synthesized AHL such as C6-HSL [7]. Cross-peaks of ROESY spectrum were clearly shown that acyl-chain of C6-HSL were interacted with α- and β-CDs. CDs are cyclic oligosaccharides, of which cavity provides lipophilic space. Consequently, AHL can be trapped by CD as host material in aqueous milieu. We expected inhibitory CD effects on prodigiosin production regulated by QS; It is reasonable that CDs in the presence of culture medium can capture AHLs diffused out from cells to form inclusion complex. Note that AHLs are considered to easily pass through cell membranes repeatedly [1].

Figure 1 showed the additive effects of α-, β-, or γ-CD on relative prodigisosin production when the *S. marcescens* was grown at 30 or 25°C. After the culture in the LB medium containing CDs for 24 h, intracellular prodigiosin was extracted to determine its amount. Since podigiosin was one of the imidazole derivatives that may be included by CD, any CDs in the system need to remove prior to cell lysis. Accordingly, the culture medium containing CD is separated first by centrifugation to avoid the complex formation between CD and prodigiosin.

Expected inhibitory effects appeared for α-, and β-CD, while γ-CD should not function as the host for AHLs because of the relatively large cavity size. Presence of α-CD made the relative prodigiosin production reduce to approximately 0.6 and 0.2 at 30 and 25°C, respectively.

NMR analysis indicated that γ-CD is not allowed to form inclusion complex with C6-HSL [7]. In the previous paper, we reported that QS inhibition was observed for the opportunistic pathogen *Pseudomonas aeruginosa* in the presence of α-, or β-CDs in the culture medium [7]. Since γ-CD addition did not affect the QS system of *P. aeruginosa*, inhibitory effects of γ-CD was unique feature solely observed for *S. marcescens*. γ-CD effects were now under investigation.

Many difficulties exist for quantitative AHL analysis among coexisted solutes in the culture medium because concentration of the signal molecule diffused out from cells is generally on the order of around 10^{-9} M or less. Although the inclusion complex formation of α- or

Table 1 Dynamic mechanical properties of hydrogel sheets at 22°C. A synthesized wave strain of 0.1 Hz was loaded during the measurements.

	E' kPa	E'' kPa	tan δ
HPC gel	27	3.1	0.12
HPC / α-CD gel	9.3	0.7	0.08
HPC / γ-CD gel	8.9	0.7	0.08
HPMC gel	14	1.3	0.09
HEC gel	17	2.0	0.12

Table 2 Relative prodigiosin production in the presence of cellulose ether gel sheets. *S. marcescens* was cultured at 30°C for 24 h.

	Relative Prodigiosin Production
Control	1.00
HPC gel	0.99
HPMC gel	1.09
HEC gel	1.01

β-CD was not directly determined in the bacterial culture medium, the results suggested that CD addition will be an effective procedure to artificially control the gene expression that regulated by QS.

3.2 Effects of CD immobilized gel on prodigiosin production

Water soluble cellulose ethers were selected for CD immobilized matrix. Since cellulose ether hydrogel was easily prepared in aqueous milieu, contamination of any organic solvent derived from gel synthesis process can be avoided during the culture. CD could be immobilized on polymer-networks at the same time as the cross-linking reaction of polymers. In this research, γ-CD besides most effective α-CD was immobilized by covalent bonds to three-dimensional polymer networks. α-CD or γ-CD easily dissolved to prepare 3.5 wt% of pre-gel solution, while β-CD solubility in water was too low to immobilize the sufficient amount of β-CD on gels.

Storage and loss moduli were evaluated to be obvious the viscoelastic properties of synthesized gel sheets (Table 1). CD immobilization made the storage modulus of HPC gel decrease because of decrement of the effective cross-linking between polymer network. Part of additive DVS was consumed not to crosslink polymers but to immobilize CD molecules. Anyway, all gel sheets possessed enough strength for the experiments and maintained the gel state during immersion in the bacterial culture solution.

Table 2 shows the effects of various cellulose ether gel sheets on relative prodigiosin production. Five gel sheets were immersed in 4 m*l* of LB medium and then 1% preculture was inoculated. No difference of prodigiosin production was observed in the presence of any gels as compared with that in the absence of gel. This result means that non-specific AHL adsorption on cellulose ether gels can be neglected to affect the

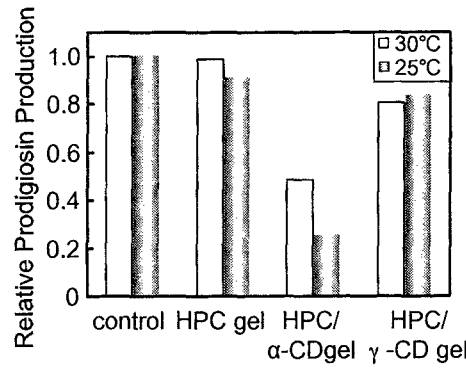

Fig. 2 Effects of cyclodextrin immobilized gels on relative prodigiosin production in *S. marcescens*. *S. marcescens* was grown at 30 or 25°C in the presence of five gel sheets in the liquid medium .

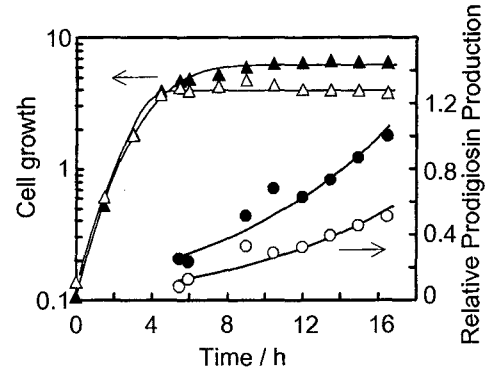

Fig. 3 Time dependence of cell growth and relative prodigiosin production for *S. marcescens*. *S. marcescens* was grown at 30°C in the absence (open symbol) or presence (closed symbol) of five HPC/α-CD gel sheets in the liquid medium: Cell growth (△), relative prodigiosin produvtion(○).

complex formation between AHL and receptor in cells. Also, cell growth was not affected by cellulose ethers (data not shown). These results clearly showed that cellulose ether is one of the suitable polymer matrices for preparation of CD immobilized gel sheet with reducing non-specific AHL adsorption onto gel sheets.

Immobilized α-CD and γ-CD showed the inhibitory effects on relative prodigiosin production (Fig. 2). As expected, α-CD was more effective than γ-CD for both 25 and 30°C. This result means that immobilized CD molecules within three-dimensional polymer network were easily interacted with diffusible AHLs and form inclusion complex. Diffusion of low molecular solute through crosslinked HPC gel sheets at swollen state was characterized as the diffusion coefficient on the order of 10^{-6}–10^{-7} cm^2/s [13]. Therefore, diffusible AHL was allowed to invade into a 1.5-millimeter-thick gel sheet during 24 h culture.

Figure 3 shows time evolution of cell growth and relative prodigiosin production in the presence of α-CD immobilized gels sheets. Cell growth was determined as turbidity of the culture solution at 600 nm (OD_{600}). After

logarithmic growth, gradual increase of prodigiosin accumulation was observed. Prodigiosin production values were normalized by that at 16.5 h in the absence of gel sheets. This result clearly showed that relative prodigiosin production was allowed to reduce to approximately 0.5 for the culture at 30°C.

4. CONCLUSION

To artificially control the gram-negative bacterial quorum sensing, a novel and effective method was proposed. Hydrogel sheets that immobilized host compounds for the signal molecule effectively reduced the QS regulated prodigiosin production in *S. marcescens*. Since non-specific AHL adsorption on cellulose ether gels did not affect the prodigiosin production, drastic decrement of relative prodigiosin production was probably responsible for the inclusion complex formation between CDs inside gels and AHLs. Immobilized α-CD on HPC gel sheets decreased prodigiosin production up to approximately 26% at 25°C. This result indicated that CD gels have high potential of controlling QS by trapping bacterium signal molecules for cell-cell communication.

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