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Uptake of Signal Molecules for Gram-Negative Bacterial Cell-Cell Communication on Polymer Gels

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Ionized polymers or polymer gels in the culture medium effectively inhibit a quorum sensing (QS) system of *Serratia marcescens*. The effects were multiplied when ionized gel sheets or polymers were coexisted with hydroxypropyl-β-cyclodextrin (HP-β-CD). Various bacteria possess a regulatory system for controlling gene expression in response to increment of cell density named as QS, of which signal molecules are *N*-acylhomoserine lactones (AHLs). The gel-induced QS inhibition was evaluated by amounts of an intracellular pigment prodigiosin that was controlled by AHL-mediated QS system. Intracellular prodigiosin was extracted from the lysate and determined by absorbance measurement after *S. marcescens* was cultured for 24 h in the presence or absence of polymer gel sheets in liquid medium. Relative prodigiosin production could be decreased to approximately 10% of the control by using the poly(acrylic acid) gel sheets and HP-β-CD. AHL molecules are easily transported to outside and inside cells and its concentration inside cells is the key for regulation of specific gene expression. Since polymer gel sheets and CDs probably adsorb the signal molecule AHL, low AHL concentration below the threshold value was responsible for the inhibitory control of QS.
Key words: quorum sensing, cell-cell communication, cyclodextrin, polymer gel, bacterial signal molecule

1. INTRODUCTION

Serratia marcescens is known to produce red pigment, 2-methyl-3-phenyl-6-methoxyprodigiosin, via quorum sensing (QS) [1,2]. Cell-to-cell communication mechanism for both gram-positive and -negative bacteria is named as QS that regulates the specific gene expression in response to cell density increase. In recent years, various QS signaling molecules were reported; Gram-positive bacteria typically use peptides, while gram-negaitve bacteria including *S. marcescens* produce acylaed homoserine lactones (AHLs) [3].

Since many pathogens regulate the virulence gene transcription via AHL-controlled QS [4, 5], artificial regulation of bacterial QS will become one of the most important preventive for virulent disease. The methods include competitive QS inhibition with synthesized antagonists [6-8] and enzymatic quenching for QS-dependent bacterial infection using acylhomeserine lactonase [9]. The principle is based on the inhibition of complex formation between AHL and its receptor protein in cells. The complex formation triggers to activate the specific gene transcription. Accordingly, AHL removal is also expected to effectively inhibit AHL-controlled gene expression.

In this research, effective AHL removal using polymer gel matrices was investigated. Since concentration magnitude of signaling molecules diffused outside of cells is generally around 10^{-9} M or less, many difficulties exist for quantitative AHL analysis among coexisted solutes in the culture medium. That's why we selected *S. marcescens*, of which QS-regulated prodigiosin production would be a useful index for activation of sequential QS process.

To regulate AHL concentration in the culture solution, inclusion complex formation between AHL and cyclodextrins (CDs) was investigated in our previous report [10]. CDs are cyclic oligosaccharides, of which molecular structure approximates a truncated cone. The molecule generates a hydrophilic exterior and a hydrophobic cavity interior that can interact with appropriately sized molecules due to hydrophobic interaction. Since acyl-chains of AHLs can be included into α- or β-CD [10], effects of additive CD were also studied in respect of prodigiosin production.

The purpose of this research is to screen the effective hydrogel matrices for AHL uptake. Magnitude of QS inhibition was elucidated by measuring intracellular prodigiosin amount in *S. marcescens*. Improvement of AHL uptake efficiency was expected by using ionized polymers; effects of electrostatic interaction between ionized polymers and AHLs were studied concerning AHL adsorption onto gel network. Moreover, multiplier effects between CD dissolved in the culture medium and electronic charge of polymers were investigated to maximize the additive effects. We evaluated cross-linked vinyl polymers and polysaccharides, including nonioninc poly(*N*-isopropylacrylamide) (NIPA), anionic poly(acrylic acid) (AAc), and cationic poly(methacryloyloxyethyl trimethylammonium) (MT MA), nonionic methyl cellulose (MC), and cationic chitosan.

2. EXPERIMENTAL

2.1 Materials

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Acros. MC, of which molecular weight is ca. 86,000 (40,000 cps for 2 wt% aqueous solution), was purchased from Sigma. NIPA, chitosan500 (300-700 cps for 5 g dm^{-3} at 20°C), chitosan1000 (800-1,500 cps for 5 g dm^{-3} at 20°C), and MTMA were purchased from Wako Pure chemical. AAc was purchased from Kanto Kagaku. All other chemicals were of reagent grade.

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

	E' kPa	E'' kPa	tan δ
NIPA gel	55	4.7	0.09
AAc gel	14	2.7	0.20
MTMA gel	81	8.9	0.11
MC gel	57	6.5	0.11
Chitosan500 gel	25	6.1	0.25
Chitosan1000 gel	14	3.7	0.26

2.2 Bacterial strain and culture conditions

S. marcescens isolated from an aeration tank of activated sludge was grown at 30°C in Luria-Bertani (LB) medium with or without 10 mM HP- β-CD for 24 h. To elucidate the AHL adsorption onto hydrogel sheets, five gel sheets (10 × 10 × 1.5 mm^3) were immersed in four m*l* of liquid medium, and then 1% of *S. marcescens* preculture was inoculated. Similarly, the liquid medium including desired amount of chitosan polymer was cultured after 1% innoulation.

2.3 Gel synthesis

Hydogels of poly(NIPA), poly(AAc), and poly (MTMA) were crosslinked with 4 mol% *N, N'*-methylenediacrylamide. After addition of ammonium peroxodisulfate and *N, N, N', N'*-tetramethylethylenediamine, the slab-shaped hydrogels were prepared between two glass plates separated by silicone rubber gasket (1.5 mm thick).

MC gel was synthesized by the method described in the previous paper [11]. Dry HPC powder (7.0 wt%) were dissolved in NaOH solution (pH 12). After 3.0 wt% of divinyl sulfone was mixed, the solution was stirred thoroughly for 30 s and poured into the mold (1.5 mm thick). The reaction was carried out at 22°C for 24h.

Chitosan gel synthesis followed the basic scheme reported in the previous paper [12].

2.4 Dynamic mechanical measurements

Dynamic mechanical measurement was carried out with slab-shaped gels to determine storage modulus *E'*, loss modulus *E''*, and loss angle (tanδ = E''/ E') by thermomechanical analyzer (Seiko Instrument, TMA/ SS6100). A synthesized wave stress (0.1 Hz) was loaded on the gel sheet with measuring the wave strain. Any measurements were carried out with the equilibrated gel sheets at 22°C. The strain was kept within 1% of the gel thickness.

2.5 Assay of prodigiosin production

Inhibitory effects of gel sheets were determined by prodigiosin amount extracted from the lysate. The relative prodigiosin production was determined by the following method [13]. *S. marcescens* was grown at 30°C in Luria-Bertani (LB) medium for 24 h. After centrifugation of the culture medium at 13,200 rpm, the pellet re-suspended in 1 m*l* of acidified ethanol (2% 2M HCl in ethanol) to extract the prodigiosin from cells. The

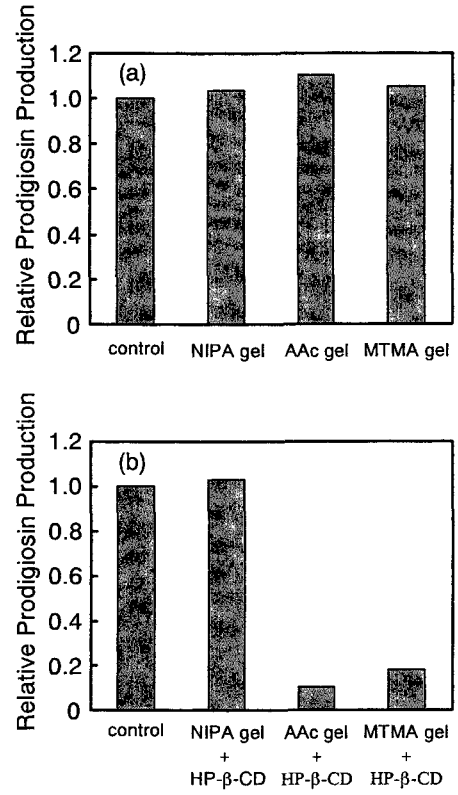

Fig. 1 (a) Effects of NIPA, AAc, or MTMA gel sheet immersion in the culture medium on relative prodigisoin production. (b) Additional effects of 10 mM HP-β-CD besides polymer gel sheets on relative prodigiosin production in *S. marcescens*. *S. marcescens* was grown at 30°C in the liquid medium for 24 h.

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). Also, cell growth was evaluated by the turbidity of culture solution at 600 nm (OD_{600}). Prodigiosin production was calculated per cell as the ratio of A_{534} and OD_{600}. The relative prodigiosin production was calculated as the index of QS regulation, while that of control without CD or gels equals to one.

3. RESULTS AND DISCUSSION

3.1 Multiplier effects between electronic charge of polymers and HP-β-CD

Table 1 shows the results for storage modulus, loss modulus, and tan δ of various gel sheets at swollen state of 22°C. All gel sheets had enough mechanical strength for the shaking culture. Also, dissolution or disintegration was not observed for any gel sheets; all gel sheets kept the gel-state even after 24 h culture in the LB medium. Note that no inhibition of cell growth was observed due to immersion of gel sheets or CD addition (data not shown).

Prodigiosin production in *S. marcescens* was determined after the cell growth in the presence of various cross-linked vinyl polymer gels including anionic AAc and cationic MTMA (Fig. 1a). No

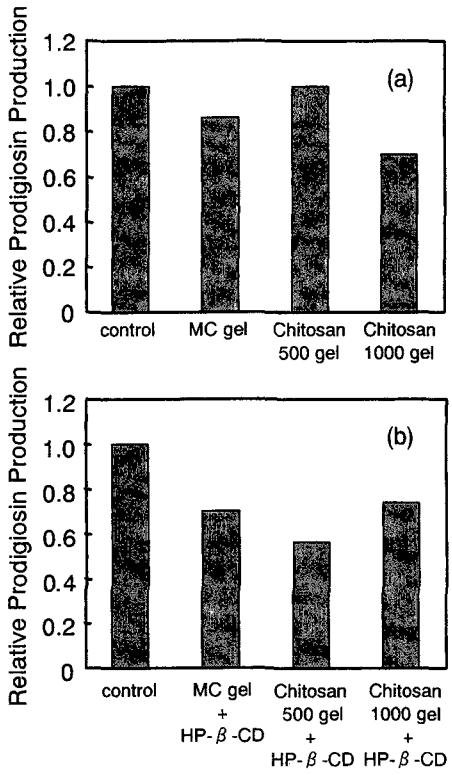

Fig. 2 (a) Effects of methyl cellulose or chitosan gel sheet immersion in the culture medium on relative prodigiosin production. (b) Additional effects of 10 mM HP-β-CD besides polymer gel sheets on relative prodigiosin production in *S. marcescens*. *S. marcescens* was grown at 30°C in the liquid medium for 24 h.

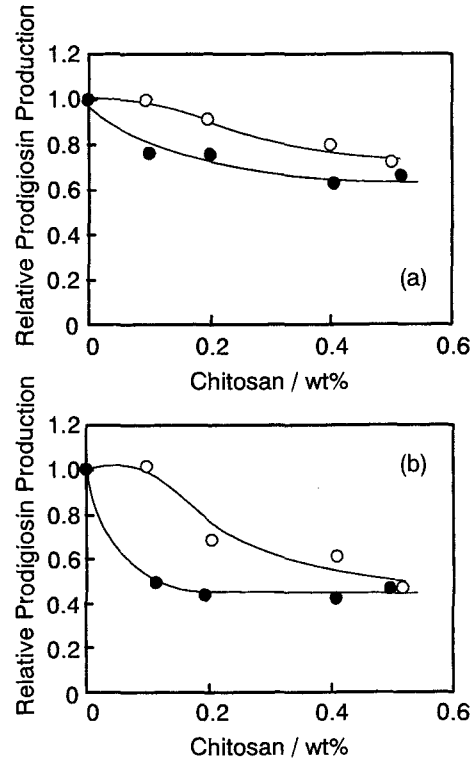

Fig. 3 Dependence of chitosan polymer concentration on relative prodigisoin production. Powder of chitosan500 (a) or chitosan1000 (b) is suspended in the culture medium during the cell growth. Relative prodigiosin production was evaluated in the medium containing desired amount of chitosan with (●) or without 10 mM HP-β-CD (○). *S. marcescens* was grown at 30°C in the liquid medium for 24 h.

differences of relative prodigiosin production were observed irrespective of polymer charges. This result means that non-specific AHL adsorption onto polymer networks was negligible for QS inhibition. Therefore, CD addition was expected to improve the inhibitory effects of prodigiosin production.

At least four AHLs were known to produce by *S. marcescens*: *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) [14]. In our previous paper, AHLs were chemically synthesized by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. 1D ^{1}H-NMR and ROESY spectra showed that AHLs such as C6-HSL were easily included to α- or β-CDs in an aqueous solution [6]. Since it was known that poor water solubility of β-CD is easily improved by chemical modification, water soluble HP-β-CD was selected as the host compounds for AHLs in this research.

Figure 1b showed the multiplier effects between cross-linked vinyl polymer gels and HP-β-CD on relative prodigiosin production. Drastic decrease of prodigiosin production was observed for anionic poly(AAc) gel and cationinc poly(MTMA) gel, while no effects appeared for noninonic poly(NIPA) gel. Because the cell growth was independent of HP-β-CD addition, destruction of cell membranes or cell growth inhibition was not caused by additives. AHLs were considered to effectively interact with HP-β-CD cavity in the presence of ionized gel. A possible explanation is that the inclusion complex is stabilized by the electrostatic interaction between polymer charges and hydrophilic moieties of AHLs. This result meant that effective AHL uptake was independent of signs of polymer charges. Note that the relative prodigiosin production reduced to 0.89 when *S. marcescens* was grown without any gel sheets in the culture medium containing 10 mM HP-β-CD.

3.2 Effects of cationic polysaccharide on relative prodigiosin production

Influence of ionized polymers onto QS inhibition was investigated by using cross-linked polysaccharide gels. Since the sign of electronic charges on polymers was independent on the effects in the case of cross-linked vinyl polymers, cationic chitosan was selected as representative ionized polysaccharides. Nonionic MC and cationic chitosan gel sheets were immersed into the liquid medium during *S. marcescens* culture. As shown in Fig. 2a, slight decrease of relative prodigiosin production indicated that AHL could be interacted to

trap inside gels even for nonionic MC gels. To evaluate the multiplier effects between gels and CD, 10 mM HP-β-CD was added to the medium. Figure 2b showed that the multiplier effects were observed for both chiosan gel sheets, of which average molecular weights were different.

To identify the interaction between AHLs and chitosan, different amounts of chitosan polymer was coexisted in the culture medium. Chitosan powder does not generally dissolve well in water but in diluted acetic acid or formic acid solution. Therefore, chitosan polymer was suspended in the LB medium during the cell growth. For the prodigiosin assay with gel sheets, chitosan contents in the liquid medium is set to be 0.2 wt% as the dry polymer. Accordingly, chitosan polymer contents varied from 0 to 0.5 wt% in the presence or absence of 10 mM HP-β-CD.

Since relative prodigiosin production decreased with increasing chitosan contents for both chitosan500 and 1000, polymer is also effective on AHL removal from the culture medium as well as the cross-linked gels (Fig. 3). Also, this result clearly shows that high molecular chitosan1000 is more effective than chitosan500. Besides the sole chitosan effects, co-existence of HP-β-CD multiplied to inhibit the QS system. According to the NMR analysis in our previous report, the binding constant between AHL and β-CD was supposed to be 10^2 M^{-1} or less. As the AHL–CD complex is not so stable in aqueous milieu, ionized polymer possibly plays important role for the complex stabilization due to interaction with the complex. Interaction between AHLs and chitosan polymer will be studied by using CD immobilized chitosan gel sheets in the next report.

4. CONCLUSION

HP-β-CD inhibitedly regulated the prodigiosin production under the control of AHL mediated QS in *S. marcescens*. The effects were multiplied when ionized gel sheets or polymers were coexisted in the culture medium besides HP-β-CD. Since both anionic AAc and cationic MTMA gel sheets possessed the similar effects, the sign of electronic charges on polymers was independent of the effects. Relative prodigiosin production could be decreased to approximately 10% of the control in the presence of poly(acrylic acid) gel sheets and HP-β-CD. Inclusion complex was probably formed between AHLs and CDs, and stabilized by the electrostatic interaction with ionized polymers. Combination of ionized polymer gels and CD could enhance the AHL removal from culture medium and reduce the gene expression that is controlled by QS.

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