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Control of Gram-Negative Bacterial Quorum Sensing with Cyclodextrin Immobilized Cellulose Ether Gel

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Abstract

Inclusion complex between cyclodextrins (CDs) and bacterial signal molecules is responsible for inhibitory effects on quorum sensing (QS). Since many bacteria have QS system for controlling gene expression in response to cell population density by means of signal molecules, an intercept of the QS signal onto the CDs can be a general method to control transcription of the QS-regulated genes. The QS in the opportunistic pathogen *Serratia marcescens* could be artificially controlled by trapping the QS signal *N*-acylhomoserine lactones (AHLs) onto the CDs because AHLs are known to work as a common QS signal in gram negative bacteria. After *S. marcescens* was cultured in the presence of 10 mM 2−hydroxypropyl-β-CD (HP-β-CD), the relative prodigiosin production could be reduced to approximately 0.86. Nonspecific adsorption of AHLs onto the cellulose ether gel sheets was very little because the relative production of prodigiosin remained 0.95–1.00 after the immersion of the gel sheets in the liquid medium throughout the cell culture. Addition of both anionic carboxymethyl cellulose gel sheets and 10 mM HP-β-CD in the culture medium effectively controlled the relative prodigiosin production to approximately 0.56. This result suggested that the ionic interaction between acceptor molecules and the AHLs could stabilize the inclusion complex and then block the sequential QS-regulated process. Moreover, CD-immobilized polymer gel sheets have high potential for the QS regulation because HP-β-CD-immobilized hydroxypropyl cellulose or hydroxypropyl methyl cellulose gel sheets effectively controlled the prodigiosin production.

Introduction

Cell-to-cell communication by means of signal molecules is important for adaptation of bacteria to the surroundings. Quorum sensing (QS) is the mechanism that allows bacteria to perceive the cell population density and respond to the information by controlling gene expression [1]. It is known that the QS system is regulated by three different-type of signals; *N*-acylhomoserine lactone (AHL) and small peptide respectively are produced in gram-negative and -positive bacteria, while production of the autoinducer-2 (AI-2) was known as a universal signal for responding to bacterial populations in both gram-negative and -positive bacteria [2].

In this research, artificial control of the AHL-mediated QS system was investigated. Each bacterial cell produces a basic level of AHLs that are allowed to be transported inside and outside the cell membrane by diffusion or active transportation. When AHL concentration increases and reaches to a threshold due to accumulation of AHL derived from each bacterial cell, bacteria can sense only a considerable amount of increased population density and respond to the information as follows. Under such high AHL concentration, a complex between a transcription factor and AHL molecule becomes stable in cells and initiates expression of particular gene. The QS system often regulates expression of virulence factors in several human pathogens, formation of biofilms, etc [3, 4].

For a variety of bacterial functions, the AHL often acts as the general key material for the QS-regulated systems. Accordingly, a novel technique for artificial control of the AHL concentration is to be expected as the preventive method for problems caused by bacteria. Some methods have been reported to control the QS system, including competitive QS inhibition with

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synthesized antagonists and enzymatic quenching with acylhomoserine lactonase [5, 6]. The method of AHL trapping on acceptor molecules is simple and effective way of keeping low level AHLs. Therefore, inclusion complex formation of AHLs and cyclodextrins (CDs) in culture broth was investigated in this research. In our previous report, 1D ^{1}H-NMR and ROESY spectra indicated that the acyl-chain of the AHL could be included into α- or β-CD cavities in aqueous media because the CDs are cyclic oligosaccharides of which interior cavity could interact with the hydrophobic moieties by hydrophobic interaction [7].

Serratia marcescens is one of the opportunistic pathogens that regulate production of the red pigment, 2-methyl-3-pentyl-6-methoxy prodigiosin, via AHL-mediated QS system [8]. This antibacterial tripyrrole material is known to appear only in the late stage of bacterial growth and accumulate in cells. No influence of the prodigiosin production on cell growth is observed as well as any sequential QS-regulated processes. Therefore, the red pigment prodigiosin could be a convenient index for *S. marcescens* to control of the QS. To evaluate the accelerated effects of soluble CDs, *S. marcescens* was grown in liquid medium in the presence of some kinds of polymer gel sheets and/or CDs. The conception of the inhibitory control of prodigiosin production in *S. marcescens* is illustrated in Figure 1, when the CD molecules were used as the AHL acceptor in the liquid medium. The purpose of this research is to screen the effective polymer matrices for inhibitory control of the prodigiosin production in *S. marcescens*. Accelerated effects between CDs and polymers on the QS control were investigated as the effective AHL trapping from the culture broth.

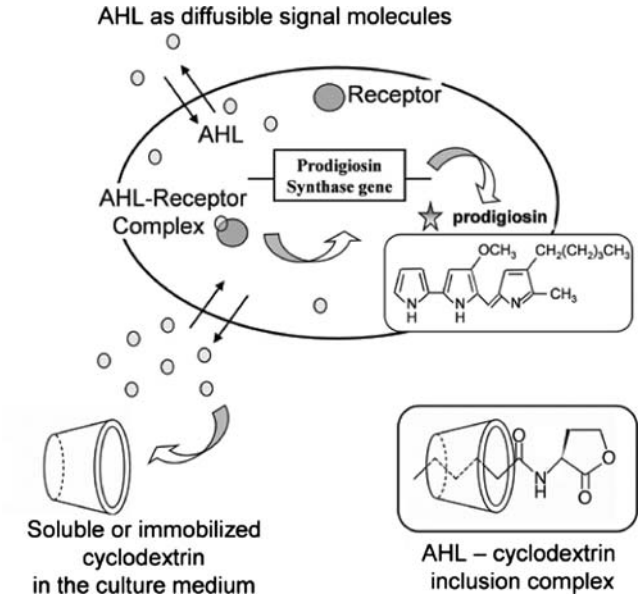

Figure 1. Control of quorum sensing in *Serratia marcescens* by trapping AHLs on cyclodextrins. The CDs are outside of cells. Transported QS signal AHLs could be included into the hydrophobic cavity of the cyclodextrins. A decrease of the local AHL concentration is responsible for blocking the sequential QS process.

Experimental

Materials

Hydroxypropyl cellulose, HPC (Mw 100,000), carboxymethyl cellulose sodium salt, CMC (Mw 90,000, DS 0.7), and 2-hydroxypropyl-β-CD, HP-β-CD (DS 3) were purchased from Acros Organics. Hydroxypropyl methyl cellulose, HPMC (Mw10,000), was a commercial product as Methocel-E (Dow Chemical). All other chemicals were of reagent grade.

Bacterial strain and culture condition

S. marcescens was grown at 30 °C in Luria-Bertani (LB) medium in the presence or absence of 10 mM HP-β-CD. To screen the suitable polymer gel sheets for CD immobilization, *S. marcescens* was cultured in 4 ml of LB medium with immersing five polymer gel sheets ($10\times10\times1.5$ mm^3). Moreover, HP-β-CD-immobilized gel sheets were immersed in the culture medium during the cell growth for 17–24 h.

Preparation of gel sheets

HPC polymer (7.0 wt%) was dissolved in distilled water and then the solution pH was adjusted to 12 by NaOH solution. After adding divinyl sulfone (1.3 wt%), the pre-gel solution was allowed to gel by placing in between two glass plates separated by silicone rubber gasket at 22 °C for 24 h [9]. Like HPC gel, HPMC or CMC gel sheets were also synthesized. Furthermore, the HP-β-CD could be immobilized on HPC or HPMC gel sheets at the time of cross-linking of the polymer. At first, 3.5 wt% HP-β-CD and 3.5 wt% polymer (HPC or HPMC) were dissolved in distilled water and pH was adjusted to 12. Then DVS (3.0 wt%) was mixed and then stirred for 30 s. The mixture was poured into the mold at 22 °C for 24 h incubation.

The prepared gel sheets were cut into pieces of $10\times10\times1.5$ mm in size and the gel pieces were fully washed with diluted HCl solution and distilled water. Non-immobilized CDs were allowed to diffuse out to the washed solution. The CD concentration in washed solution was determined by HPLC refractive index measurement (Shimadzu RID-6A). The concentration of CD leakage into the washed solution indicated that more than 95% CD in the pre-gel solution could be immobilized on the gel sheets.

Prodigiosin assay

S. marcescens was grown in LB liquid medium with immersing five gel sheets in a shaker at 30 °C for 17–24 h. After the separation of gel sheets and cells, intracellular prodigiosin was extracted from the cells in acidified ethanol solution (4% 1 M HCl in ethanol) [10]. The prodigiosin production was determined as the ratio of the absorbance of the extracted prodigi-

osin solution at 534 nm to turbidity of the culture suspension as OD_{600}. Effects of CDs and gel sheets were evaluated as the relative prodigiosin production, of which control sample without any CDs or any gel sheets equals to one.

Results and discussion

Effects of soluble HP-β-CD and cellulose ethers on QS control

Different bacterial species produce different AHLs which vary in length of the acyl chains. In *S. marcescens*, four classes of AHLs were known as the QS signal; *N*-hexanoyl-, *N*-heptanoyl-, *N*-octanoyl-, and *N*-(3-oxohexanoyl)-homoserine lactones are abbreviated as C6-HSL, C7-HSL, C8-HSL, and 3-oxo-C6-HSL, respectively [11]. While some classes of AHLs possessing the different acyl-chains are operated, the signals contain the same homoserine lactone moiety in their molecules. Although the exact role of individual AHL is still not known, effective capture of such AHL signals onto CD or polymer gel sheets is responsible for blocking expression of the QS-regulated gene (Figure 1). To create the preventive materials for the QS-dependent gene transcription, the acceptor molecule for the AHL signal is expected to be immobilized on some kinds of polymer films. Therefore, HP-*β*-CD and cellulose ether were dissolved in LB medium prior to the inoculation of *S. marcescens*. Since the CDs and such cellulose ether polymers respectively are oligo- and polysaccharides which were constructed by the same glucopyranose unit and the same 2-hydroxypropyl groups, HPC and HPMC were selected for this study.

Figure 2 shows the effects of HP-*β*-CD and cellulose ether polymers on relative prodigiosin production. According to the OD_{600} measurements, presence of the CD and cellulose ethers did not affect the cell growth. Prodigiosin displays a characteristic absorption spectrum in extracted solution with maximum at 534 nm. The relative prodigiosin production could be reduced to approximately 0.86 when 10 mM HP-*β*-CD was dissolved in the LB medium. In the presence of 26 mg of HPC or HPMC polymer in 4 ml of the LB medium besides 10 mM HP-*β*-CD, the relative prodigiosin production decreased to 0.71 and 0.65, respectively. Note that five HPC or HPMC gel sheets (10×10×1.5 mm) contains approximately the same amount of polymers in the following experiments. This result shows that the coexistence of cellulose ether polymers and HP-*β*-CD in the system has advantage for AHL capture.

Effects of cellulose ether gel sheets and soluble HP-β-CD on QS control

The chemically crosslinked CMC gel, HPC gel, HPMC gel were prepared by divinyl sulfone. Each gel sheet was immersed in the LB medium containing 10 mM HP-*β*-CD and then 1% of *S. marcescens* fresh culture was inoculated and incubated in a shake culture at 30 °C for 24 h. The extracted prodigiosin was determined by measuring the absorbance. The cell growth is independent of the gel sheet immersion. Moreover, no dissolution and disintegration of the gel sheets were observed even after the cell culture for 24 h. Accelerated effects on the prodigiosin production were noticed when the cellulose ether gel sheets and HP-*β*-CD were coexisted in the medium rather than the medium provided with only HP-*β*-CD (Figure 3). Among the three kinds of gel sheets, anionic CMC gel sheet was found to be more effective to control the prodigiosin production. Non-specific adsorption of AHLs on CMC gel sheets was negligible because the drastic decrease of the relative prodigiosin production was not observed when only

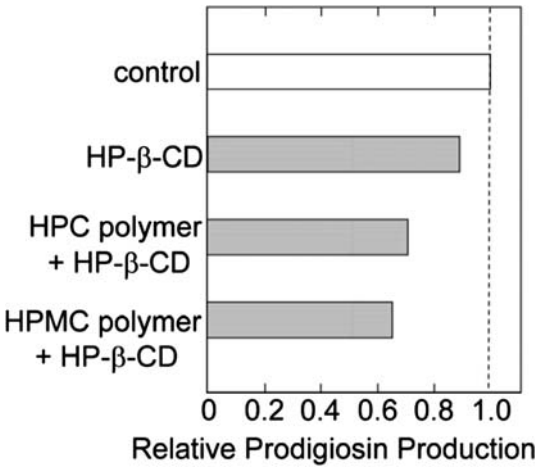

Figure 2. Control of the relative prodigiosin production with addition of soluble cyclodextrins and cellulose ether polymers.

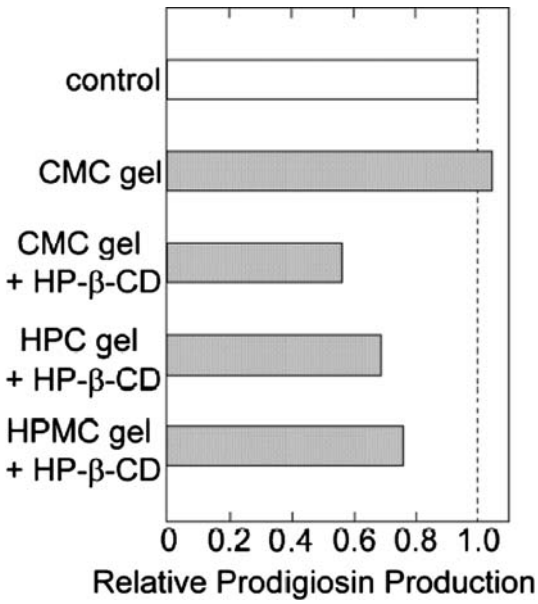

Figure 3. Control of the relative prodigiosin production with addition of soluble cyclodextrins and cellulose ether gel sheets.

CMC gel sheet was added. Although more study is to be needed for better understanding of the effects of the ionic interaction between additives and AHLs, but at present the possible explanation could be given as the inclusion complex of the CD becomes stable in the presence of ionized polymer in the system.

Effects of HP-β-CD immobilized gel sheets on QS control

Prodigiosin assay was carried out after the cell growth in the presence of HP-β-CD-immobilized gel sheets. Five pieces of gel sheets for HPC or HPMC contained approximately 17 μmol of HP-β-CD; *i.e.*, it corresponded to approximately 4 mM HP-β-CD in the liquid medium. In our previous report, inclusion complex formation in aqueous media was investigated with chemically synthesized AHL analogues and CDs. It appeared that the inclusion complex binding constant of AHL onto β-CD analogues was estimated to below 10^3 M^{-1} from the results of NMR analyses. Since the AHL concentration in cells is supposed to be in the order of 10^{-9} mol l^{-1}, 10^{-3} mol l^{-1} level of the CDs is probably indispensable to effectively trap AHLs according to the binding constant. As shown in Figure 4, HP-β-CD-immobilized HPC or HPMC gel sheets could effectively reduce the relative prodigiosin production to approximately 0.7. These effects of immobilized CD were comparable to those of soluble CDs in Figures 2 and 3. This result clearly indicated that CD immobilization onto the polymer films or gel sheets does not lose its ability of AHL capturing and CD-immobilized polymer matrices have high potential for materials to control the bacterial QS system.

Time evolution of cell growth and relative prodigiosin production was studied in the presence or absence of HP-β-CD-immobilized HPC gel sheets for 17 h (Figure 5). The value of relative prodigiosin production for the control sample without gel sheets was defined as 1.0 at 17-h culture. According to the cell growth curve, a stationary phase appeared at around 6-h culture. No

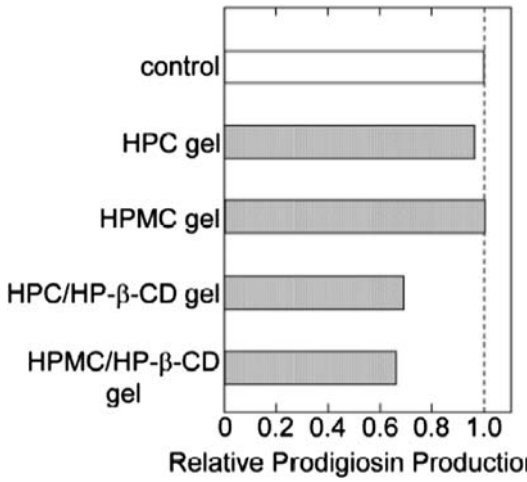

Figure 4. Control of the relative prodigiosin production with addition of cyclodextrin immobilized cellulose ether gel sheets.

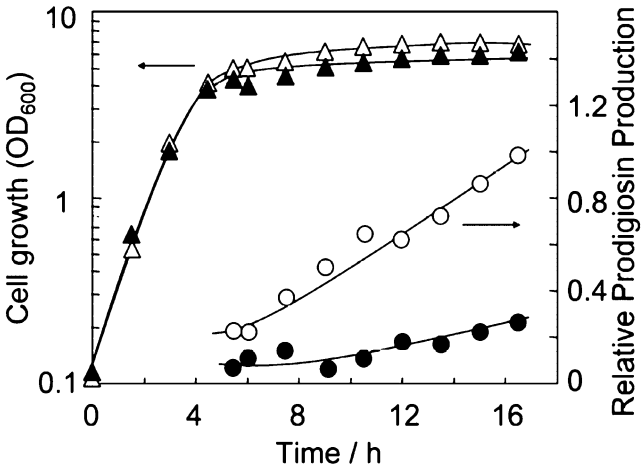

Figure 5. Time evolution of the relative prodigiosin production and cell growth. Cell growth was plotted as OD_{600} of the culture suspension. The relative prodigiosin production was calculated as the ratio of absorbance at 534 nm of the extracted prodigiosin solution to cell growth (OD_{600}), while the relative prodigiosin production value for the control sample without gel sheets was defined as zero at 17-h culture. *S. marcescens* was grown at 30 °C in the LB liquid medium with (closed symbol) or without (open symbol) five pieces of HP-β-CD-immobilized HPC gel sheets: Cell growth (Δ), relative prodigiosin production (○).

significant differences of the cell growth were observed when the HP-β-CD-immobilized gel sheets were coexisted in the LB medium throughout the cell growth. This figure clearly showed that the CD-immobilized gel sheets effectively maintain low level of prodigiosin and the result could be interpreted as the trapped AHLs onto the CD-immobilized gels probably caused to block the sequential process of QS-regulated gene expression.

Conclusion

Cyclodextrins could operate as the AHL acceptor to form the inclusion complex in the culture broth. Intracellular red pigment prodigiosin production could be reduced by adding soluble CDs or immobilized CDs on cellulose ether gel sheets. The nonspecific adsorption of AHLs on the HPC, HPMC, or CMC was found to be negligible and decrease of the prodigiosin production indicated that QS signal AHL was probably trapped in the hydrophobic cavity of the CD. The CD effects on inhibitory control of the QS system was accelerated by coexistence of soluble cellulose ether polymers or chemically crosslinked gel sheets. The inclusion complex between CDs and AHLs possibly became stable by adding cellulose ether polymers especially by ionic carboxymethyl cellulose.

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