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Improved bioassay for detecting autoinducer of *Rhodovulum sulfidophilum*

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Abstract. Quorum sensing is a bacterial gene regulation system that enables prompt environmental adaptation in response to cell density. Quorum sensing is driven by an extracellularly secreted chemical signal called autoinducer. Gram-negative bacteria produce one or several types of *N*-acylhomoserine lactone (AHL) as autoinducers. Our previous study suggests that the gram-negative marine photosynthetic bacterium Rhodovulum sulfidophilum produces AHL in the early stationary phase and plays a role in maintaining the bacterial cell aggregates called "floc". We performed conventional bioassay to identify AHL production by using Chromobacterium violaceum VIR07, which produces violet pigment (violacein) in response to AHL with side chains ranging from C10 to C18 in length. However, we were not able to observe the violacein with good reproducibility, suggesting that inhibitory chemical compounds co-existed in the AHL extract. Therefore, we improved the extraction method; the ethyl acetate-extracted AHLs were fractionated by using reverse phase TLC. By using the re-extracted AHLs for the bioassay, we observed an obvious production of violacein. This result clearly indicates that R. sulfidophilum produces AHLs with side chains ranging from C10 to C18 in length and suggests the utility of improved bioassay for AHL detection.

INTRODUCTION

Quorum sensing is an intracellular chemical communication system first discovered in *Viblio fischeri* bioluminescent luciferase production (1). This system is now widely observed among gram-negative and gram-positive bacteria (2). Quorum sensing is controlled by extracellularly produced chemical signals referred to as autoinducer. Bacteria can detect the concentration of extracellular autoinducers in order to know how many of the same species or different species are around the bacteria. In response to the concentration of autoinducer, the quorum sensing related gene is expressed or repressed to adapt environmental exchanges (2).

In gram-negative bacteria, the well-studied common autoinducer is *N*-acylhomoserine lactone (AHL) (Fig. 1). AHL has several types of acyl-chains from C4 to C18 in length, and each gram-negative bacterium expresses species specific AHLs as autoinducers. The Ikeda group at Utsunomiya University shows that genetically engineered *Chromobacterium violaceum* VIR07 can use AHL detecting bacterium (3). This bacterium produces a violet pigment (violacein) in response to AHL with side chains ranging from C10 to C18 in length while the violacein production is inhibited in the presence of short chain AHLs ranging from C4 to C8 in length. Therefore, *C. violaceum* VIR07 is widely used for AHL detection.

The gram-negative marine photosynthetic bacterium Rhodovulum sulfidophilum produces either DNA or RNA extracellularly, and the extracellular DNA and RNA play an important role in forming cell aggregates called "floc" (4). Our previous study revealed that alpha-cyclodextrin, which held AHL in the hydrophilic sugar ring and inhibited quorum sensing, induced suppression of the floc maintenance (4). This result implied that *R. sulfidophilum* produced AHL and the quorum sensing system of R. sulfidophilum affected the floc maintenance in the stationary

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phase. In this report we improved the bioassay using *C. violaceum* VIR07 to obtain direct evidence that *R. sulfidophilum* produced AHL.

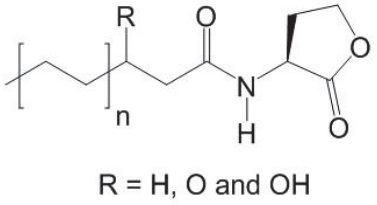

FIGURE 1. Structure of *N*-acylhomoserine lactone (AHL), popular AI in gram-negative bacteria.

MATERIALS AND METHODS

Strains

Bacterial strains *Chromobacterium violaceum* VIR07 (3) and CV026 (5), were kindly provided by Prof. Ikeda and Dr. Morohoshi (Utsunomiya University), Prof. Williams (Univeristy of Nottingham), respectively. *Chromobacterium violaceum* Bergonzini 1880 (NBRC no. 12614) and *Rhodovulum sulfidophilum* strains (DSM1374 and DSM2351) were obtained from the National Institute of Technology and Evaluation (Japan) and Leibniz Institute DSMZ-German Collection of Microorganism and Cell Culture (Germany), respectively.

Extraction of AHLs

Chromobacterium violacein strains were grown to a stationary phase in LB broth. *Rhodovulum sulfidophilum* strains were grown as described by Suzuki *et al*. (4). In brief, *R. sulfidophilum* strains were cultivated under either anaerobic dark conditions or aerobic light conditions in the nutritionally rich medium, PYSM broth, or nutritionally poor medium, M5-M broth. After cell cultivation, cells were removed by centrifugation and the recovered supernatant was filtrated through a 0.45 mm pore-sized membrane filter. The culture filtrates were extracted with equal volumes of ethyl acetate. The organic phases were pooled, and residual water was removed by addition of anhydrous Na_2SO_4, and the organic phases evaporated to dryness on a rotary evaporator. Residues were dissolved in 50 µl of ethyl acetate and stored at –20 °C.

Determination of the violacein production (agar plate assay)

For the agar plate assay, LB broth containing 0.7% agar mixed with a 1% (v/v) overnight culture of either *C. violaceum* VIR07 or CV026 was poured onto the 1% agar plates. A 3MM disk filter paper was soaked with 30 µl volumes of ethyl acetate-extracted AHLs from the culture of *R. sulfidophilum* and air dried. The air-dried 3 MM disk filter was placed on the agar plate.

For the control experiment, 1 µl of 5.0 nM *N*-hexanoyl-L-homoserine lactone (C6-HSL) or 3.9 nM *N*-decanoyl-L-homoserine lactone (C10-HSL) and 1 µl of ethyl acetate-extracted AHLs from the culture of *C. violaceum* Bergonzini 1880 (NBRC 12614) were used for the agar plate assay. Assay plates were incubated overnight at 30 °C, and violacein production from *C. vioraceium* VIR07 and CV036 was monitored.

Improved bioassay using thin-layer chromatography

Ethyl acetate-extracted AHLs were fractionated by using RP-C18 reverse phase TLC (Silica gel 60 RP-18F_{254}S, MERC) and developed with 90% (v/v) methanol in water. For estimating AHLs fractions, C6-HSL, C10-HSL, C14-HSL and C18-HSL (Funakoshi, Japan) were used as standards and each retention factor (R_f) was estimated by using phosphomolybdic acid. According to the R_f value of each standards, TLC was fractionated into eight fractions and re-extracted with ethyl acetate. The re-extracted AHLs were then applied to a 3MM disk filter paper and placed onto

LB agar plate that was covered with 0.7% agar mixed with 1% (v/v) overnight culture of *C. violaceum* VIR07 or CV026. The assay plates were incubated overnight at 30 °C.

RESULTS AND DISCUSSION

For determination of AHL production from Rhodovulum sulfidophilum, we extracted AHL supernatants at the stationary phase with ethyl acetate, and brown extracts were obtained (see disk filter shown in Figure 2). The extracts were dissolved in the small portion of ethyl acetate and used for the conventional bioassay. In this experiment, violacein production was very low and the cell growth inhibition ring around the disk filter paper was observed on occasion. Therefore, we assumed that the AHL extracts from *R. sulfidophilum* contained inhibitory compounds for violacein production of *Chromobacterium violaceum*.

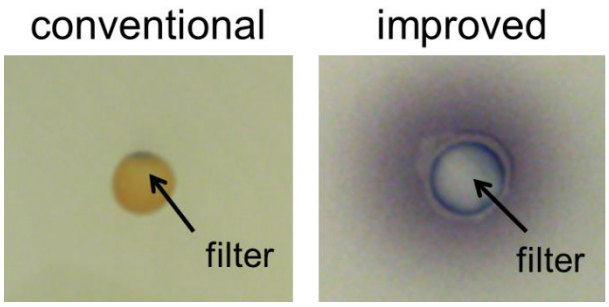

FIGURE 2. Comparison between the conventional assay and the improved assay. Shown are the results of the conventional assay (left) and the improved assay (right). Arrows indicate disk filter. The right panel shows the violet pigment production surrounding the disk filter.

TABLE 1. Summary of violacein detection by conventional and improved bioassay. "+", "±" and "–" denote the detected, slightly detected and not detected violacein, respectively. "Rich" and "Poor" indicates PYSM medium and M5-M medium, respectively. Fraction number 2, 4, 6 and 8 correspond to the standards fractions of C6-HSL, C10-HSL, C-14-HSL and C18-HSL, respectively.

Extracts	Incubation conditions	Medium conditions	Fraction number							
			1	2	3	4	5	6	7	8
R. sulfidophilum DSM 1374	anaerobic light	rich	–	–	–	±	+	+	+	+
		poor	–	–	–	±	±	±	±	+
	aerobic dark	rich	–	–	–	+	+	+	+	+
		poor	–	–	–	±	±	+	±	±
R. sulfidophilum DSM 2351	anaerobic light	rich	–	–	–	+	+	+	+	+
		poor	–	–	–	±	±	±	±	+
	aerobic dark	rich	–	–	–	±	+	±	+	+
		poor	–	–	–	+	±	+	±	±

To reduce cell inhibitory compounds in the ethyl acetate extracted AHL fraction, we conducted reverse phase TLC. According to the R_f values of AHL standards, developed TLC was fractionated into eight sections and re-extracted with ethyl acetate. Using the re-extracted fractions, violacein production was clearly detected (Figure 2). This result indicated that the ethyl acetate supernatants contained AHLs and some inhibitory compounds were removed by the re-extraction process.

Next we observed AHL production from *R. sulfidophilum* under several cultivating conditions. This phototrophic bacterium can grow in both aerobic and anaerobic conditions (4); also, cultivation conditions may affect metabolic pathways including quorum sensing pathways. Therefore, we prepared four combinations of cultivation conditions: such as anaerobic dark or aerobic dark conditions in nutritionally rich medium (PYSM medium) (4) or nutritionally poor medium (M5-M medium) (4). While the conventional bioassay did not detect AHL production (except for aerobic dark conditions in the poor medium), the improved assay was able to detect AHLs from all cultivation conditions (Table 1). Furthermore, AHL was detected from fraction 4 to fraction 8, which corresponded to the AHL standard fraction of C10-AHL, C14-AHL and C18-AHL, respectively. These data indicated that *R. sulfidophilum* produced several AHLs with side chains ranging from C10 to C18 in length (Table 1).

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