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Development of a Sensitive Bioassay Method for Quorum Sensing Inhibitor Screening Using a Recombinant *Agrobacterium tumefaciens*

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Abstract Acylhomoserine lactones (AHLs) are known to be the triggering molecules in the quorum sensing mechanism of many gram-negative bacteria. In order to detect AHL inhibitors that are potential biofilm inhibitors, a convenient and sensitive bioassay was developed based on the β-galactosidase activity (β-GAL) of a recombinant *Agrobacterium tumefaciens* strain. A series of commercially available AHLs were tested for inducing β-GAL at varying concentrations in agar-plate and liquid cultures of the reporter strain. All AHLs tested exhibited a concentration-dependent induction, and octanoyl homoserine lactone (OHL) showed the highest sensitivity with a detection limit of 0.1 nM in the liquid culture assay. When fimbrolide, a known quorum sensing inhibitor, was added, induction of β-GAL by OHL was repressed. The repression at a constant OHL concentration was dependent on the fimbrolide concentration with the detection limit below 1 ppm, indicating that this assay is a sensitive method for screening AHL inhibitors.

Keywords: quorum sensing inhibitor, bioassay, acyl homoserine lactones, *Agrobacterium tumefaciens*, fimbrolide, biofilm inhibitor

INTRODUCTION

Bacterial biofilms are found in many places in nature including the human body. They are of great concern because they can result in diseases or cause environmental hazards [1,2]. The cells in biofilms are more resistant to antibiotics than their planktonic counterpart due to the expression of various protective genes including that for the formation of an exopolymer lipopolysaccharide [3,4]. Biofilms are known to be formed through a unique mechanism called "quorum sensing" which involves membrane-permeant signaling molecules [5]. In gram-negative bacteria, most of these compounds are acylated homoserine lactones (AHLs) and their analogues which interact with transcriptional activator protein to couple gene expression with cell population density [6,7]. In gram-positive bacteria, γ-butyrolactones and post-translationally modified peptides are also involved in quorum sensing [8].

Inhibitors of quorum sensing signaling molecules could be developed into biofilm inhibitors. Some AHL analogues have already been reported to inhibit the quorum sensing mechanism by interfering with the function of AHL through competitive binding to the transcriptional activator protein [9]. Other AHL inhibitors are produced by bacterial species that interact with plants as well as by non-bacterial species like the marine red alga, *Delisea pulchra* from which halogenated furanone analogues, fimbrolides, were isolated [10,11]. Moreover, Teplitski *et al.* [12] recently reported that exudates of pea seedlings exhibit stimulation and inhibition of AHL-regulated actions. The identification of these AHL inhibitors in non-bacterial species may be possible in other forms of natural products as well. The problem, however, is that bioactive secondary metabolites are usually present at very low concentrations with various interfering compounds. Thus, developing a sensitive and selective bioassay seems to be an important prerequisite in the search of biofilm inhibitors.

Several bioassays to detect AHLs have been developed [9,13-16]. These methods rely on the presence of a reporter gene whose expression is under the control of the AHL-transcriptional activator protein complex. When incubated in a proper medium, the reporter strain produces luminescence [17] or color in response to the presence of AHLs. A recent study revealed an assay where AHLs could be detected at concentrations of pico molar levels using TLC [18]. However, the focus of most of these detection methods has been in showing the existence of various AHLs in different bacterial cultures to prove their stimulating actions in the quorum sensing mechanisms [19]. Very few studies report the application

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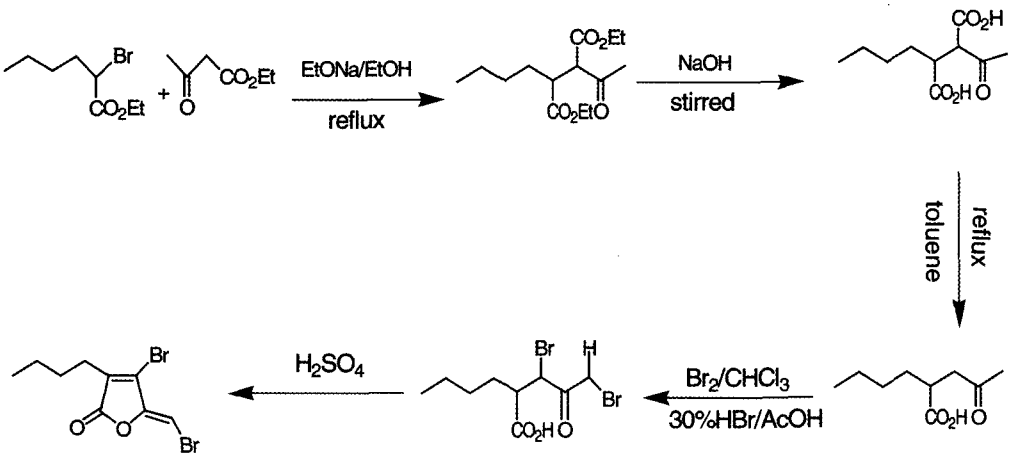

Fig. 1. Synthetic scheme of fimbrolide.

of these assays in screening AHL inhibitors [20].

The present study focuses on developing a highly sensitive bioassay for detecting AHL inhibitors in gram-negative bacteria that may be later developed into biofilm inhibitors. The attempt has been made by modifying and optimizing an AHL detection assay in a quantitative manner. The recombinant *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) was selected as the reporter strain because its β-galactosidase activity that responded to a AHL-transcriptional activator protein complex could be induced by a wide variety of AHLs [9]. Herein we report on the effect of the kind and concentration of AHL autoinducers on quorum sensing response and determination of the optimum conditions including the AHL detection limit of the bioassay. Further verification by testing a synthetic fimbrolide analogue which is a known AHL inhibitor and evaluation of the feasibility of applying the bioassay to natural products screening have also been attempted.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains *A. tumefaciens* NTL4 (pCF218) (pCF372) and *A. tumefaciens* KYC6 (pCF218) were kindly provided by Dr. Fuqua at Indiana University, USA [21]. The strain NTL4 was used as a reporter strain for detecting AHL autoinducers or AHL inhibitors. The genetic element pCF218 (Tc^R, tetracycline resistant and Km^R, kanamycin resistant) coded for the transcriptional activator protein, traR, while the pCF372 (Sp^R, spectinomycin resistant) harbored *lacZ* reporter under *traI* promoter that was activated by the presence of traR-AHL complex. The KYC6 strain was an over-producer of an AHL autoinducer, *N*-(3-oxooctanoyly)-HSL and harbored *traM* null mutation with *traR* plasmid (pCF218). Prior to use, both strains were incubated at 30°C on AT minimal agar plates (1.5%) supplemented with Tc (4.5 μg/mL) and Sp (50 μg/mL) for strain NTL4 and with Km (100 μg/mL) and Tc (4.5 μg/mL) for strain KYC6. The AT minimal medium contained KH_2PO_4 (0.079 M), NaOH (0.044 M), $(NH_4)_2SO_4$ (0.015 M), $MgSO_4 \cdot 7H_2O$ (0.6 mM), $CaCl_2 \cdot 2H_2O$ (0.06 mM), $FeSO_4 \cdot 7H_2O$ (0.027 mM), $MnSO_4 \cdot H_2O$ (0.0071 mM), and 0.5% glucose.

Preparation of AHL Derivatives and X-gal Solution

The AHLs used in this study were the non-substituted analogues which were commercially available (Fluka): *N*-hexanoyl-DL-homoserine lactone (HHL), *N*-octanoyl-DL-homoserine lactone (OHL), *N*-decanoyl-DL-homoserine lactone (DHL), *N*-dodecanoyl-DL-homoserine lactone (DDHL) and *N*-tetradecanoyl-DL-homoserine lactone (TDHL). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma) and AHLs were dissolved in DMF (dimethylformamide, Sigma) and/or methanol (Aldrich) and filtered through a 0.2-μm Acrodisc syringe filter (Gelman). They were stored in a -70°C deep freezer before use.

Synthesis of 4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (Fimbrolide Standard)

4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone was synthesized following the procedure of Manny *et al.*'s [22] as shown in Fig. 1. Ethylacetoacetate (Aldrich, 2.0 g, 15.4 mmole) and ethyl-2-bromohexanone (Aldrich, 3.4 g, 15.4 mmole) were reacted to obtain the 2-acetyl-3-butylbutanedioate. Hydrolysis, decarboxylation, bromination and cyclization followed to give a mixture of furanone analogues. Careful chromatographic separation using silica gel as stationary phase and cyclohexane as mobile phase yielded 336 mg (21%) of the desired fimbrolide whose structure was identified by ^1H and ^{13}C NMR (Varian 300) and IR (Mattson GL-3020).

Plate Bioassay for the Detection of AHLs with Recombinant *A. tumefaciens*

When *A. tumefaciens* NTL4 is used as the reporter

strain, the expression of both *traR* and *lacZ* is induced by adding AHLs and the level of induction can be measured by monitoring the hydrolysis of the chromogenic substrate X-gal through the action of β-GAL. The cross-feeding bioassay by McLean *et al.* was modified to achieve better sensitivity and reproducibility [23,24]. LB agar (including 1.5% agar) was poured into a 45-mm diameter petri dish and dried overnight in a 30°C incubator. The agar plate was spread evenly with 20 μL of 20 mg/mL X-gal solution after which the AHL solution was streaked. Instead of streaking the NTL4 strain approximately 1 cm distant from the AHL (as described in the cross-feeding bioassay by McLean *et al.* [23]), the reporter strain was overlaid on top of the AHL to reduce the amount of AHLs being trapped in the agar during the diffusion through the agar matrix. After 24 h incubation at 30°C, the intensity of the blue color was examined. Activation of the *traI–lacZ* fusion in the reporter strain resulted in the blue coloration due to hydrolysis of X-gal by β-galactosidase (β-GAL) activity. Positive and negative controls consisted of the reporter strain with and without the AHL-overproducing strain KYC6, respectively.

Liquid Culture Assay with Recombinant *A. tumefaciens*

The liquid culture assay was a modification of the plate bioassay, measuring the AHL activity using the absorbance mode of the Victor^2 Multilabel Counter (1420-011, Wallac, Finland). The *A. tumefaciens* NTL4 was cultivated in AT minimal medium with Sp (50 μg/mL) and Tc (4.5 μg/mL) overnight at 30°C, under aerobic condition. The overnight culture was then inoculated in 1:10 fresh AT minimal medium and grown to an O.D. (at 600 nm) of 0.9~1.3. Afterwards, the cells were diluted in 1:50 using fresh AT minimal medium and taken to a sterile 96-well plate (270 μL) along with X-gal solution (to reach a final concentration of 40 μg/mL) and additional AHL solutions (30 μL). Concentrations of AHLs ranged from 10^3 to 10^{-3} nM. Experiments were conducted with orbital shaking for 6 h 40 min at 30°C. At 10 min intervals, the intensity of blue color in each well was measured continuously at 600 nm. When inhibitors of AHLs were used in the assay, cells in AT minimal medium (240 μL), AHLs (30 μL) and inhibitors (30 μL) were added to bring the final volume of the well to be 300 μL. The inhibitors tested were fimbrolide synthesized in the present study and a marine natural product supplemented with the fimbrolide. The inhibitor solutions were prepared after calculating the final doses needed for the assay. The marine natural product was a methanol extract of a Korean red alga obtained from the Marine Natural Products laboratory of Dongseo University.

Capacity Factor Determination by Reversed-phase HPLC

A modified HPLC analysis method by Michels *et al.* was used [25]. A Waters 2690 liquid chromatograph equipped with a diode array UV detector was used for obtaining the capacity factor, k', for AHLs used in this study. One mg of AHL was dissolved in 100 mL of HPLC grade methanol and each sample was injected (20 μL) on a Lichrospher® 100 CN (5 μM) column at a flow rate of 1.0 mL/min employing a mobile phase of 10% acetonitrile/5 mM H_3PO_4. The detector wavelength was 210 nm. The mobile phase hold-up time, t_0, was determined by injecting the mobile phase. Each sample was injected 3 times and the average of the 3 retention times (t_R) was used in calculating k' from the following equation [26]:

$$k' = \frac{t_R - t_0}{t_0}$$

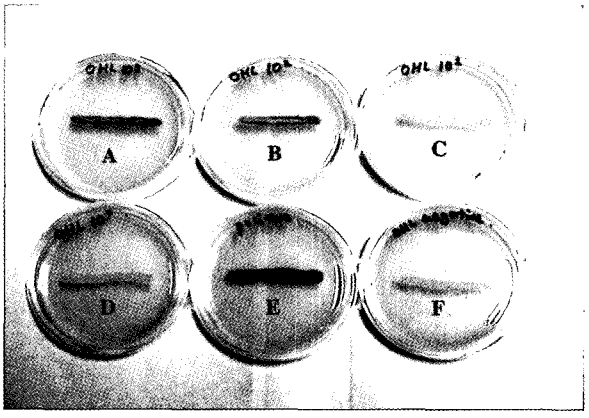

Fig. 2. Plate bioassay. Color response of β-galactosidase activity of the reporter strain was shown with varying concentrations of OHL [10^3 nM (A), 10^2 nM (B), 10 nM (C) and 1 nM (D)], AHL-producing *A. tumefaciens* KYC6 (pCF218) (E), or DMF as control (F).

RESULTS AND DISCUSSION

Recombinant *A. tumefaciens* Plate Bioassay to Detect AHLs

Many autoinducers identified in gram-negative as well as gram-positive bacteria are analogues of the simple acylhomoserine lactone. The bioassay methods used for detecting AHLs can be modified for detecting inhibitors of AHLs. If the response of a reporter strain to a specific AHL is known in a quantitative manner, the reduction of the response by the presence of an inhibitor can also be determined. This indicates that natural products can be screened for the presence of AHL inhibitor by incubating them with a reporter strain and a proper amount of AHL. Before attempting the detection of AHL inhibitors, the conditions for detecting AHLs have been examined in the plate bioassay in a quantitative manner. A series of parameter studies have revealed that the optimal conditions for the plate bioassay were: 40 μg/mL of X-gal solution; 24 h incubation time; overlaid-streaking of the sample or AHL-overproducing strain *A. tumefaciens* KYC6 on the reporter strain in a 45-mm petri dish. For OHL, the blue color of the reporter strain increased with sequentially increasing concentration (Fig. 2). The detection limit,

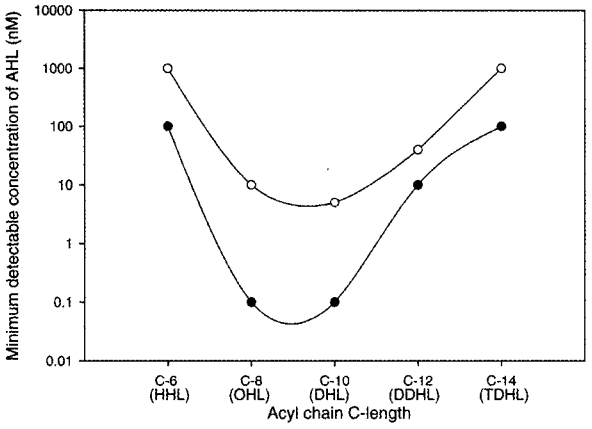

Fig. 3. Effect of different AHLs on their minimum detectable concentration in the plate bioassay (○) and liquid culture assay (●). Carbon number of acyl chain refers to the number of carbons of the acyl chains of each AHL derivative. Each dot represents the mean ± S.D. of 3 independent experiments.

Table 1. Capacity factor (k')* of the AHL analogues tested

AHL analogue (Carbon number of acyl chain)	HHL (C-6)	OHL (C-8)	DHL (C-10)	DDHL (C-12)
k'	0.51	3.94	18.18	63.92

* Determined by retention times of reversed phase HPLC analysis. TDHL was too lipophilic to be analyzed at the same condition.

which was the lowest concentration that gave a discernible blue color response compared to the negative control, varied with alkyl chain lengths; it decreased up to carbon number 8~10 in AHL side chain then increased thereafter (Fig. 3). The OHL exhibited the highest sensitivity with its detection limit as approximately 10 nM (Fig. 2).

HPLC Analysis of AHLs and Their Capacity Factors

Sensitivity of the bioassay was affected by the acyl carbon chain length of the AHLs, which implies that lipophilicity of AHLs may affect their transport through cell membrane and/or binding affinity to the receptor. The difference in capacity factor in the HPLC analysis can be representative of the relative lipophilicity [27] suggesting that an optimum lipophilicity exists for interaction. Using a CN column instead of the C-18 column gave well-separated peaks of distinguishable retention times. OHL was detected at about 6.4 min while HHL at about 3.4 min, and the longer chained analogues DHL and DDHL at about 28.4 min and 92.8 min respectively (data not shown). For TDHL, the retention time was too long going beyond 2 h and thus an accurate retention time could not be determined. Capacity factors, k', calculated based on the HPLC retention times are shown in Table 1. The importance of hydrophobicity of a bioactive compound has been well recognized in drug development studies, as it is crucial in crossing the cell membrane as well as in receptor interactions. A parabolic relationship between

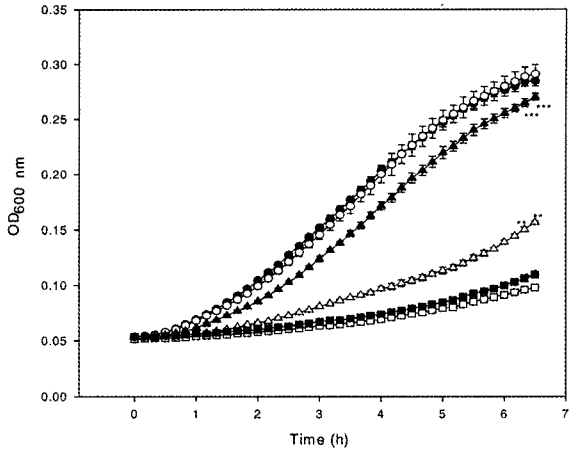

Fig. 4. Liquid culture assay with *A. tumefaciens* NTL4. Change of optical density at 600 nm was measured with varying amount of OHLs: (●), 10^5 nM; (○), 10^3 nM; (▲), 10 nM; (△), 1 nM; (■), 10^{-1} nM; and (□), 0 nM. Each dot represents the mean ± S.D of 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control.

biological activity and hydrophobic character has been documented, indicating that extreme hydrophobicity can cause poor aqueous solubility [28]. This also seemed to be the case in the present bioassay employing *A. tumefaciens*. However, the difference in acyl chain length of AHLs may also affect the binding between AHL and the transcription activator protein (TAP) and/or the binding of AHL-TAP complex to the promoter site, which are closely related to the expression of the β-GAL and the minimal detection limits of AHLs. Further studies are underway to examine the relationship between these two factors and the acyl chain length of AHLs.

Liquid Culture Bioassay to Detect AHLs

The limitation of the plate bioassay is that decision on color change has to be made solely based on the observation with the naked eye. This means that an objective decision is difficult when the coloration or disappearance of coloration is at minimum level. Moreover, the use of petri dishes and the sample volume required in a large quantity are inconvenient or even inappropriate for high throughput screening. Therefore, the plate assay was modified into a liquid culture assay where the blue coloration could be quantitatively determined by spectrophotometric analysis at 600 nm. Using a 96 well plate, the assay was downsized so that larger numbers of samples could be simultaneously analyzed. Fig. 4 shows the change of OD_{600} with time at different OHL concentrations in a typical liquid culture assay. The optical density increased with time by blue coloration as well as by cell growth. However, compared to β-GAL activity, the OD increase by cell growth was only marginal in the wide range of OHL since minimal growth medium was used (see the control run without OHL in Fig. 4). In addition, the relative contributions of β-GAL activity and cell growth to the increase of OD

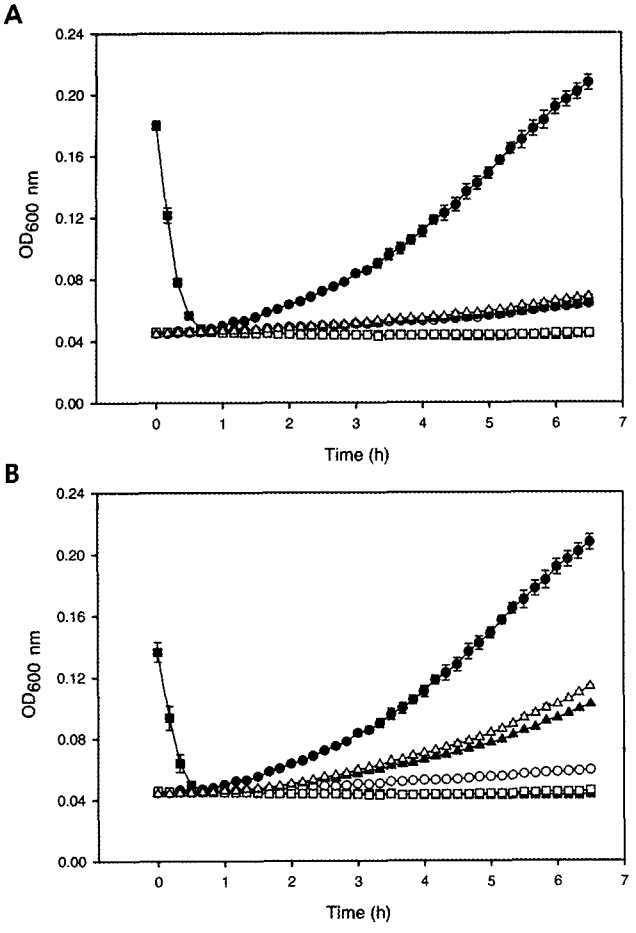

Fig. 5. Liquid culture assay with *A. tumefaciens* NTL4 for measuring the inhibition of OHL activity by fimbrolide. Change of optical density at 600 nm was measured with varying amount of fimbrolide without OHL (A) and with 10^3 nM OHL (B). Fimbrolide concentration were: (△), 0.1 ppm; (▲), 1.0 ppm; (□), 10 ppm; and (■), 100 ppm. Control experiments were conducted with 10^3 nM OHL (●) or 1% methanol (○), each without fimbrolide. Each dot represents the mean ± S.D of 3 independent experiments.

values were easily discernable. The extent of coloration was dependent on OHL added and increased up to 10^3 nM. The detection limit was determined as the smallest concentration at which a statistical significance (*t* test, $p<0.05$) was observed between the absorbance values of the samples and the negative control at 6 h. As shown in Fig. 4, the detection limit for OHL was 10^{-1} nM. For other AHLs, similar experiments were conducted and the detection limits were determined. As shown in Fig. 3, they were; 10^{-1} nM for DHL and 10^2 nM for HHL or TDHL. Noticeably, for most AHLs tested, the liquid culture assay gave a significantly improved sensitivity by about 100 fold compared to the plate bioassay mentioned above.

The plate bioassay employing the recombinant *A. tumefaciens* NTL4 (pCF218)(pCF372) strain was reported to detect a wide range of structurally related AHL analogues [9]. By modifying the assay into a liquid culture system, less amount of sample was required and, furthermore, more accurate and quantitative analysis was possible. This assay is, therefore, an appealing bioassay that can be further developed into a high throughput screening system for AHLs and AHL inhibitors. It would also constitute an attractive bioassay for bioactivity-guided fractionations of natural products.

Liquid Culture Assay to Detect AHL Inhibitors: Fimbrolide as a Model AHL Inhibitor

Upon evaluation of the liquid culture assay as being sensitive, dose-dependent and simple in AHL detection, the next step was to confirm the ability of the assay to detect AHL inhibitors. Fimbrolide analogues have been isolated from the Australian red alga, *Delisea pulchra*, and their activity as quorum sensing inhibitors have been reported [10]. According to the scheme shown in Fig. 1, 4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone, one of the bioactive fimbrolide analogues has been synthesized and used as the model AHL inhibitor in this study. Since the present assay system depends on β-GAL activity, the effect of the furanone on the pure enzyme of β-GAL (Sigma) was examined in the range of 0.1 ppm and 1,000 ppm. Up to 100 ppm, the effect of the furanone was negligible; however, at 1,000 ppm, β-GAL activity was inhibited by 50% (data not shown). Fimbrolide was added to the liquid culture assay mixture with or without OHL to see its effect on color development and/or cell growth of the reporter strain (Figs. 5A and B). When OHL is not present (Fig. 5A), the effect of fimbrolide addition at 0.1 and 1.0 ppm was negligible. At higher concentrations of 10 and 100 ppm, the inhibition of cell growth was observed. At 100 ppm, an extraordinarily high initial OD was observed, which was attributable to the poor solubility of the fimbrolide in aqueous solution, but disappeared about one hour after incubation. Fig. 5B shows the inhibition of OHL activity in the presence of fimbrolide. The coloration by 10^3 nM OHL was repressed in a dose-dependent manner when fimbrolide was added. At the OHL concentration of 10^3 nM, 1 ppm of fimbrolide, corresponding to 3.36×10^3 nM, almost completely inhibited the color response by the reporter strain. Since the interaction between β-GAL (Sigma, USA) and fimbrolide was negligible below the concentration of 100 ppm of fimbrolide, the reduced absorbance in the presence of the fimbrolide is attributable to the inhibition of quorum sensing response. The present assay consisting of the reporter strain and a known amount of AHL will therefore be able to detect the presence of quorum sensing inhibitors. The similar magnitude of concentration between OHL and fimbrolide suggests that their mobility through the cell wall as well as binding affinity with TraR in the reporter strain may be comparable.

The inhibition of OHL activity by fimbrolide was further examined by varying OHL concentrations in the range of 10 and 10^5 nM (data not shown). As shown in Fig. 5B, the color development was repressed by fim-

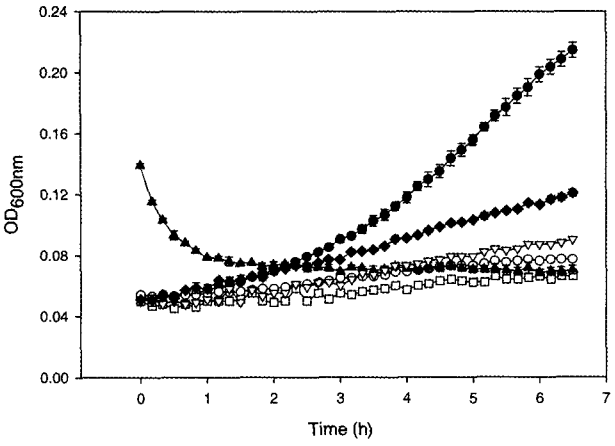

Fig. 6. Liquid culture assay with *A. tumefaciens* NTL4 for measuring the inhibition of OHL activity by fimbrolide-spiked marine natural product. *A. tumefaciens* NTL4 strains were incubated with 10^3 nM OHL and 1,000 ppm natural product extract which contained fimbrolide at concentrations of 100 ppm (▲), 10 ppm (▽), 1 ppm (◆), and 0 ppm (●). (□) represents results with 1,000 ppm natural product extract without OHL; and (○) negative control of 1% methanol and 0.01% DMF without the natural product extract.

brolide in a dose-dependent manner in all OHL concentrations tested. The concentration of fimbrolide to repress OHL activity could be decreased as OHL concentration decreased, indicating that the assay system becomes more sensitive when OHL concentration is lowered. However, optimal OHL concentration was determined as 10^2~10^3 nM since clear coloration of the assay mixture was required when it does not include an AHL inhibitor.

Further verification of the assay was made by spiking fimbrolide into the methanol extract of a marine natural product (Fig. 6). The natural product extract was originally devoid of AHL inhibitory action up to a concentration of 10^3 ppm. However, when supplemented with fimbrolide, it was able to suppress the β-GAL activity stimulated by OHL. The inhibition by the natural product extract plus fimbrolide was dependent on fimbrolide concentration and the extent or the pattern of the inhibition was similar to that by fimbrolide alone. This indicates that the presence of AHL inhibitors contained in natural products extracts can be successfully detected by this assay system. Further investigations are in progress to screen AHL inhibitors from marine as well as terrestrial natural product extracts using the bioassay system developed in this study.

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