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Inhibitory Effects of 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone (HDMF) on Acyl-Homoserine Lactone-Mediated Virulence Factor Production and Biofilm Formation in *Pseudomonas aeruginosa* PAO1

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4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), a non-halogenated furanone found in a variety of fruits, has been shown to have antimicrobial activity. However, few studies have focused on its inhibitory effect on bacterial quorum sensing (QS) at levels below the non-inhibitory concentration. In this study, 0.1 μM HDMF decreased the production of QS signal molecules and inhibited QS-controlled biofilm formation by *Pseudomonas aeruginosa* PAO1 without causing growth inhibition. In the presence of 0.1 and 1.0 μM HDMF, biofilm production by PAO1 was reduced by 27.8 and 42.6%, respectively, compared to that by untreated control cells. HDMF (1.0 μM) also significantly affected virulence factor expression (regulated by the *las*, *rhl*, and *pqs* system), resulting in a significant reduction in the production of LasA protease (53.8%), rhamnolipid (40.9%), and pyocyanin (51.4%). This HDMF-dependent inhibition of virulence factor expression was overcome by increasing the levels of two QS signal molecules of *P. aeruginosa*, N-(3-oxo-dodecanoyl)-L-homoserine lactone and N-butyryl-L-homoserine lactone, suggesting reversible competitive inhibition between HDMF and these molecules. The results of this study indicate that HDMF has great potential as an inhibitor of QS, and that it may be of value as a therapeutic agent and in biofilm control, without increasing selective pressure for resistance development.

Keywords: acyl-homoserine lactone, biofilm, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, *Pseudomonas aeruginosa*, quorum sensing

Introduction

Quorum sensing (QS) is the mechanism by which bacteria perceive the density of surrounding bacterial populations and coordinately respond to this information by regulating various genes (Smith and Iglewski, 2003a). In Gram-negative bacteria, QS usually involves the production of an acyl-homoserine lactone (AHL) as the signal. *Pseudomonas aeruginosa* has become one of the most important model organisms for studies in this field. *P. aeruginosa* is an opportunistic human pathogen that causes endocarditis, pneumonia, and catheter-related infections, and which is a serious problem among patients hospitalized with cystic fibrosis and severe burns (Dubin and Kolls, 2007). The major characteristics of *P. aeruginosa* pathogenesis include the expression of QS-controlled virulence factors (LasA protease, LasB elastase, rhamnolipid, pyoverdin, pyocyanin, and toxins) and the formation of intractable biofilms, which endow the bacterial cells with resistance to many antimicrobial agents and protection from the host defense system (Davies *et al.*, 1998). There is increasing demand for effective strategies to control virulence factor production and biofilm formation by *P. aeruginosa*, particularly with consideration to the increase in antibiotic resistance observed and the induction of biofilm formation by antibiotic use (Stewart and Costerton, 2001; Hoffman *et al.*, 2005).

As a therapeutic candidate, much interest has been shown in targeting the QS regulatory mechanism of *P. aeruginosa* due to the critical role of QS in virulence expression and biofilm formation (Smith and Iglewski, 2003b). Such regulation works through specific interference of the QS signaling system, including enzymatic degradation of AHLs (Dong *et al.*, 2001; Reimmann *et al.*, 2002), inhibition of AHL synthesis by S-adenosyl methionine analogs (Parsek *et al.*, 1999), sequestration of AHLs (Whitehead *et al.*, 2001), and inhibition of QS signal transduction by antagonistic AHL analogs competing for AHL receptors (Smith and Iglewski, 2003b).

Halogenated furanones, a class of secondary metabolites first characterized in the red alga *Delisea pulchra*, have shown considerable promise as QS inhibitory (QSI) compounds (de Nys *et al.*, 1993). These furanones were found to inhibit QS-controlled gene expression in *P. aeruginosa* by displacing the AHL signals from their cognate receptor proteins at sub-inhibitory concentrations, which is essential for avoiding selective pressure leading to the development of resistance (Manefield *et al.*, 1999; Hentzer *et al.*, 2002). Following the discovery of the halogenated furanones produced by *D. pulchra*, a variety of furanone analogs have been synthesized to enhance QSI activity and their QSI potential has been successfully evaluated against *Erwinia carotovora*, *Serratia liquefaciens*, *Vibrio fischeri*, *Chromobacterium violaceum*, and

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P. aeruginosa (Rasmussen *et al.*, 2000; Manefield *et al.*, 2001, 2002; Hentzer *et al.*, 2002; Martinelli *et al.*, 2004). However, halogenated furanones are highly reactive and thus likely too toxic to treat bacterial infections in human and animals, although their ability to control *P. aeruginosa* infections in animal models is promising (Hentzer and Givskov, 2003; Rasch *et al.*, 2004; Khan *et al.*, 2009). Therefore, there is growing need for effective and safe QSI compounds. The identification of such compounds could provide a strategy to control QS-regulated pathogenesis and to control biofilm growth in medical equipment, water pipelines, water filtration facilities, and industrial systems.

Previous studies have demonstrated that 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) exhibits anti-carcinogenic, anti-oxidative, antimicrobial, and anti-cell adhesion activities (Sasaki *et al.*, 1998; Slaughter, 1999; Manefield *et al.*, 2002; Sung *et al.*, 2007; Gule *et al.*, 2013). However, there have been no reports to date investigating the QSI activity of HDMF against bacteria at concentrations below the growth non-inhibitory concentration. HDMF, a natural organic compound found in a variety of fruits such as strawberries, pineapples, and mangos, is used extensively as a safe food additive in beverages, ice cream, and cigarettes (Sung *et al.*, 2007). In this study, we tested the QSI effect of HDMF on *P. aeruginosa* and demonstrated that HDMF efficiently inhibited the QS-dependent production of AHLs, biofilm formation, and the expression of virulence factors, including LasA protease, rhamnolipid, and pyocyanin. The results of this research may lead to the development of new strategies for the treatment of *P. aeruginosa*-related infections and biofilms encountered in various medical, industrial, and environmental processes.

Materials and Methods

Bacterial strains and culture conditions

In this study, *P. aeruginosa* PAO1 (ATCC BAA-47; ATCC, USA) was used to test the QSI activity of HDMF. PAO1 was cultured overnight (16 h) in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) at 30°C with shaking (150 rpm). For growth inhibition and AHL production assays, an overnight culture of PAO1 was diluted to an A_{600} of 0.2 with fresh LB medium containing appropriate concentrations of HDMF and incubated at 30°C with shaking. The cell densities of the PAO1 cultures were determined by measuring the A_{600} value at time intervals to evaluate growth inhibition. *Staphylococcus aureus* (ATCC 12600) was cultured overnight in LB medium at 30°C with shaking (150 rpm) and used for LasA protease bioassay. *Chromobacterium violaceum* CV026 (ATCC 31532; McClean *et al.*, 1997) and *Agrobacterium tumefaciens* NTL4 (pZLR4; Luo *et al.*, 2003) were used as biosensor strains for detecting N-butyryl-L-homoserine lactone (BHL) and N-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL). These strains were cultured in TY medium (Difco Laboratories, USA) and AB mannitol medium (ATCC medium 1691), respectively. When necessary, 50 μg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and antibiotics (500 μg/ml carbenicillin and 100 μg/ml kanamycin) were added to the AB mannitol medium. Reagent-grade HDMF, AHLs, X-Gal, and the antibiotics were all purchased from Sigma-Aldrich (USA).

The effects of HDMF on biofilm formation

The quantification of biofilm formation was performed by a microtiter plate assay as described previously (O'Toole and Kolter, 1998). An overnight culture of PAO1 was diluted to an A_{600} of 0.2 with fresh LB medium supplemented with 0.1 or 1.0 μM HDMF. Aliquots (1 ml) of each culture were placed into the 48 wells of a polystyrene microtiter plate (Falcon, BD, USA). After incubation for 24 and 48 h at 30°C, the medium was carefully removed and 1 ml of 1% crystal violet solution (Sigma-Aldrich) was added. The dye solution was removed after 20 min and the wells were rinsed thoroughly with distilled water. To quantify the attached cells, the crystal violet was solubilized in 1 ml of 95% ethanol and the A_{595} was measured. A PAO1 culture without HDMF served as a positive control; all samples were prepared in triplicate.

Biofilm formation was also assessed by scanning electron microscopy (SEM). PAO1 cultures were prepared as described above for the microtiter plate assay and placed in a 24-well plate (Falcon), each well of which contained a glass coverslip (9 × 9 mm). After 24 h of culture at 30°C, the coverslips were retrieved and rinsed twice with sterile phosphate-buffered saline (PBS, 0.1 M). The biofilm on the coverslip was fixed in glutaraldehyde (2.5%; Sigma-Aldrich) overnight, washed with PBS three times, and gradually dehydrated in a series of ethanol washes from 30 to 95%. The coverslips were then air-dried overnight, mounted on studs and coated with gold plasma, and examined by SEM (Hitachi S-3500N; Hitachi Scientific Instruments, USA). Our observations were documented through the acquisition of at least 20 representative microphotographs for each sample.

The effects of HDMF on swarming, swimming, and twitching motility

Swarming motility was tested on nutrient broth (NB) medium (0.3% beef extract and 0.5% peptone, pH 6.8 ± 0.2) supplemented with glucose (0.5%, Sigma-Aldrich) and HDMF (0, 0.1, or 1.0 μM) and solidified with 0.5% agar (Difco) (Reimmann *et al.*, 2002). Plates were dried for 15 min and inoculated with 1-μl aliquot of an overnight culture of PAO1 in NB, and then incubated at 37°C for 16 h. Swimming motility was tested on tryptone motility medium (1% tryptone, 0.5% NaCl, 0.3% agar) supplemented with HDMF (0, 0.1, or 1.0 μM) (Doyle *et al.*, 2004). Plates were dried for 30 min and inoculated with overnight culture of PAO1 on LB agar (1.5% agar) using a sterile toothpick. The diameter of the swimming motility zone was measured 13 h after incubation at 30°C. Twitching motility was tested on LB supplemented with HDMF (0, 0.1, or 1.0 μM) and solidified with 1% agar. The medium was dried for 10 min and stab-inoculated with overnight cultured PAO1 on LB agar (1.5% agar) using toothpick (Reimmann *et al.*, 2002). Plates were incubated for overnight at 37°C followed by 48 h at room temperature, and the zone of twitching motility at the Petri plate-agar interface was measured.

Virulence factor assays

Overnight cultures of PAO1 were diluted 100-fold with fresh LB medium containing HDMF (0.1 or 1.0 μM), and then incubated at 30°C for 24 h with shaking. The cultures were then centrifuged at 10,000 × *g* for 10 min and the supernatants were used for the assays after membrane filtration (0.20 μm pore size; Whatman, UK). When specified, BHL or OdDHL was added to the diluted cultures at a final concentration of 10.0 μM to investigate the mechanism of inhibition of HDMF. PAO1 cultured without HDMF served as a positive control. PAO1 cultured without HDMF and supplemented with acylase I (0.15 mg/ml, porcine kidney; Sigma-Aldrich) served as a negative control (Sio *et al.*, 2006; Paul *et al.*, 2009). Each assay was conducted in triplicate.

LasA protease activity was determined by measuring the capability of the PAO1 supernatants to lyse boiled *S. aureus* cells (Kong *et al.*, 2005). After centrifugation of the boiled *S. aureus* culture at 10,000 × *g* for 10 min, the pellet was suspended in 10 mM Na_2HPO_4 (pH 4.5) and the A_{600} was adjusted to 0.8. The prepared suspension (900 μl) was mixed with 100 μl of PAO1 supernatant and the A_{600} was monitored at intervals of 0, 5, 10, 15, 20, 30, 45, and 60 min for changes in turbidity. LasA protease activity was expressed as the average change in A_{600} value per h.

For the rhamnolipid assay (Pearson *et al.*, 1997), a 333-μl aliquot of the PAO1 supernatant was washed three times with diethyl ether (1 ml each time). After drying the ether fractions under nitrogen gas, the residue was reconstituted in 0.5 ml of deionized water. A 100-μl aliquot of each extract was mixed with 900 μl of orcinol solution (0.19% in 53% H_2SO_4; Sigma-Aldrich), heated for 30 min at 80°C, and the A_{421} was measured after cooling the sample. The concentration of rhamnolipid was expressed as mg rhamnose/L by comparing the data with rhamnose standards (L-rhamnose; Sigma-Aldrich).

For the pyocyanin assay, 1 ml of PAO1 supernatant was mixed with 1 ml of chloroform to extract the pyocyanin into the solvent. The chloroform phase was then added to 0.2 N HCl (0.2 ml), producing a pink to deep red color, indicative of the presence of pyocyanin. The absorbance was measured at 520 nm and pyocyanin concentrations were calculated using a multiplication factor of 17.072 (Essar *et al.*, 1990).

Well diffusion assay for BHL and OdDHL

PAO1 was cultured with various HDMF concentrations for 24 h and then centrifuged at 10,000 × *g* for 10 min. A 5-ml aliquot of the supernatant was extracted three times with the same volume of ethyl acetate (acidified with formic acid to a final concentration of 0.5%). The pooled ethyl acetate fraction was evaporated under nitrogen flow and the residue obtained was then reconstituted in 2 ml of acidified ethyl acetate and stored at -30°C. For the detection of BHL, an overnight culture of CV026 (200 μl) was added to 10 ml of fresh TY agar (0.8% agar) at 50°C and the agar-culture mixture was immediately poured into a Petri dish. The stored ethyl acetate extracts (300 μl) were evaporated under filter-sterilized nitrogen flow and reconstituted in 30 μl of fresh TY medium, then placed into wells (4 mm in diameter) made in the solidified medium. The plates were incubated for 24 h, and the diameter of each colored circle was measured. For OdDHL detection, the procedure was the same as for the BHL bioassay except NTL4 was used as the bioassay strain in AB mannitol agar supplemented with X-Gal (50 μg/ml). The amounts of BHL and OdDHL were calculated according to standard curves created from the relationship between the Log [moles of AHL] and the surface area of the colored circle (Ravn *et al.*, 2001).

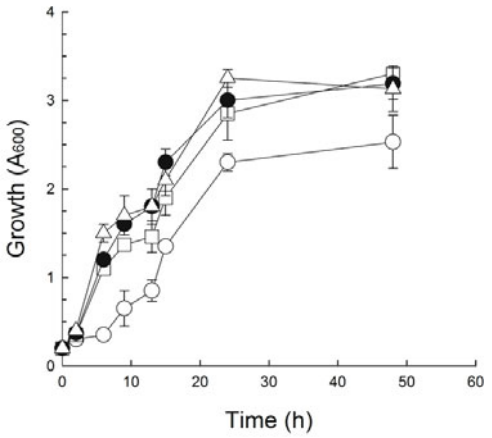

Fig. 1. The effects of HDMF on the growth of *P. aeruginosa* PAO1. (●) 0.0 μM HDMF, (△) 0.1 μM HDMF, (□) 1.0 μM HDMF, (○) 10.0 μM HDMF.

Results

The effect of HDMF on the growth of *P. aeruginosa* PAO1

A growth inhibitory effect on PAO1 was observed in the presence of 10.0 μM HDMF beginning at 6 h (Fig. 1). This concentration was much lower than the minimum inhibitory concentration (MIC) of 624.8 μM for wild-type *P. aeruginosa* (KCTC1637) and the MICs for various multi-antibiotic resistant *P. aeruginosa* strains (156.2–312.4 μM) re-

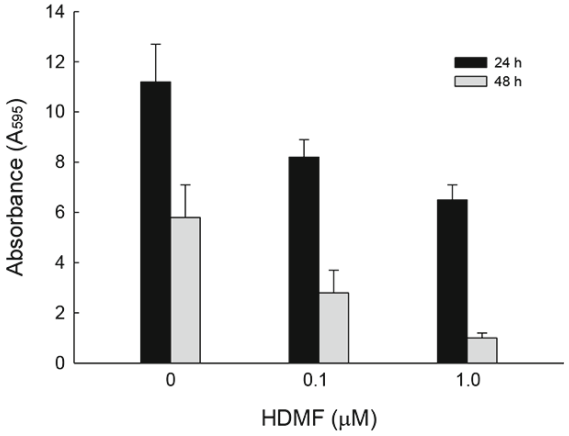

Fig. 2. The inhibition of biofilm formation by HDMF. The effects of HDMF (0.0, 0.1, and 1.0 μM) on biofilm formation by *P. aeruginosa* PAO1 was analyzed using a crystal violet microtiter plate assay.

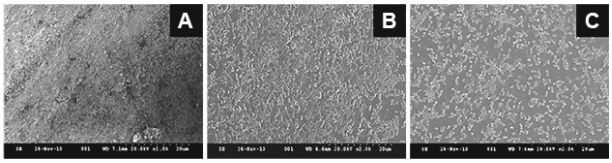

Fig. 3. The effects of HDMF on biofilm architecture. Scanning electron micrographs of *P. aeruginosa* PAO1 biofilms formed on a polystyrene surface in the presence of varying concentrations of HDMF. (A) 0.0 μM, (B) 0.1 μM, (C) 1.0 μM HDMF.

ported by Sung *et al.* (2007). The growth of PAO1 with 0.1 and 1.0 μM HDMF did not show any significant delay or acceleration as compared to the no HDMF control. Based on these results, we concluded that the QSI effect of HDMF at concentrations below 1.0 μM was not due to growth inhibition.

The inhibition of biofilm formation and motility by HDMF

HDMF efficiently inhibited PAO1 biofilm formation based on a crystal violet microtiter assay (Fig. 2). After 24 h of cultivation, biofilm formation by PAO1 was decreased by 27.8 and 42.6% in the presence of 0.1 and 1.0 μM HDMF, respectively, compared to the no HDMF control. During the stationary growth phase (24–48 h), a loss of biofilm was observed in all cultures. In cultures grown for 48 h, the reduction in biofilm formation in the control (no HDMF) was 50.4%, while that in the HDMF (0.1 and 1.0 μM) cultures was 66.3 and 84.8%, respectively. Thus, HDMF not only inhibited biofilm formation but also facilitated cell detachment from the preformed biofilm.

The thickness and architecture of the biofilm formed under increasing concentrations of HDMF exhibited obvious inhibitory effects. The biofilms formed in the presence of 0.1 and 1.0 μM HDMF (Figs. 3B and 3C) exhibited a less established architecture, and most cells were sparsely scattered on the substratum in a thin monolayer compared to the no HDMF control (Fig. 3A), which appeared mature and stacked densely in multi-cell layers.

We also tested the effect of HDMF on bacterial motility which is critical for biofilm formation in *P. aeruginosa* (Overhage *et al.*, 2007). Swarming motility was substantially decreased in the presence of 1.0 μM HDMF compared to the no HDMF control (Table 1). Although swimming zone diameter was decreased by 7.6 and 14.6% in the presence of 0.1 and 1.0 μM HDMF, respectively, the inhibition was much less compared to that on swarming. The effect of HDMF on twitching motility was only marginal regardless of the HDMF concentration.

Table 1. The effects of HDMF on the motility of *P. aeruginosa* PAO1
Swarm, swim, and twitch media were prepared to assess the corresponding type of motility in the presence or absence of HDMF. [a,b] The diameters of the swimming and twitching motility zones were measured.

Motility	HDMF (μM)		
	0.0	0.1	1.0
Swarming			
Swimming[a] (mm)	15.8 ± 1.0	14.6 ± 0.4	13.5 ± 1.1
Twitching[b] (mm)	17.8 ± 2.3	16.6 ± 1.3	16.9 ± 0.9

Table 2. The Effects of HDMF on QS-regulated virulence factors
The production of virulence factors was determined using the spent medium after 24 h of culture of *P. aeruginosa* PAO1. [a] LasA protease activity is expressed as the reduction in OD_{600} per hour; [b] Rhamnolipid production is expressed as mg/L; [c] Pyocyanin production is expressed as $OD_{520} \times 17.072$.

HDMF (μM)	Production of virulence factors		
	LasA protease[a]	Rhamnolipid[b]	Pyocyanin[c]
0.0	0.13 ± 0.01	158.80 ± 5.90	4.18 ± 0.09
0.1	0.12 ± 0.02	156.30 ± 6.30	4.22 ± 0.16
1.0	0.06 ± 0.01	93.80 ± 3.10	2.03 ± 0.20

The implications of HDMF for QS

P. aeruginosa has three QS systems (*las*, *rhl*, and *pqs*), which control the expression of a host of genes involved in virulence, biofilm formation, and the production of extracellular substances (Schaadt *et al.*, 2013). Hence, the QSI effects of HDMF may be evaluated by measuring the production levels of virulence factors such as LasA protease (*las*), rhamnolipid (*rhl*), and pyocyanin (*pqs*).

Regardless of the HDMF concentration, there was no significant difference in LasA protease activity or rhamnolipid and pyocyanin production in PAO1 cultures until 9 h of incubation (data not shown). A marginal inhibitory effect of HDMF (0.1 μM) was observed for all virulence factors after 24 h of incubation (Table 2). However, in the presence of 1.0 μM HDMF, the production of LasA protease, rhamnolipid, and pyocyanin was substantially reduced to 53.8, 40.9, and 51.4%, respectively, compared to the control (no HDMF).

P. aeruginosa produces two AHLs as QS signaling molecules, OdDHL and BHL. OdDHL and BHL are the cognate signaling molecules for the *las* and *rhl* systems, respectively (Schaadt *et al.*, 2013). A well diffusion assay (Steindler and Venturi, 2007) was employed to determine the concentrations of BHL and OdDHL produced by *P. aeruginosa* PAO1 using *C. violaceum* CV026 and *A. tumefaciens* NTL4 as biosensors for BHL and OdDHL, respectively. These results indicate that the production of BHL and OdDHL decreased in an HDMF concentration-dependent manner (Table 3). In the presence of 0.1 μM HDMF, there was a 54.7 and 55.4% reduction in BHL and OdDHL production, respectively. The addition of 1.0 μM HDMF resulted in a significant decrease in BHL (85.9%) and OdDHL (92.8%) levels, suggesting that at this concentration HDMF may severely interfere with the quorum induction of the *las* and *rhl* system in *P. aeruginosa*.

To evaluate whether the HDMF-induced inhibition of QS induction could be overcome by the exogenous addition of AHLs, PAO1 cultures were exposed to BHL or OdDHL at a concentration of 10 μM, which is the saturation concentration for QS signal induction in *P. aeruginosa* (Hentzer *et al.*, 2002; Kim *et al.*, 2008). The addition of 10 μM OdDHL significantly enhanced LasA protease activity and rhamnolipid production to levels greater than those obtained without the addition of HDMF and AHL (dotted lines in Fig. 4). The addition of 10 μM BHL also increased rhamnolipid pro-

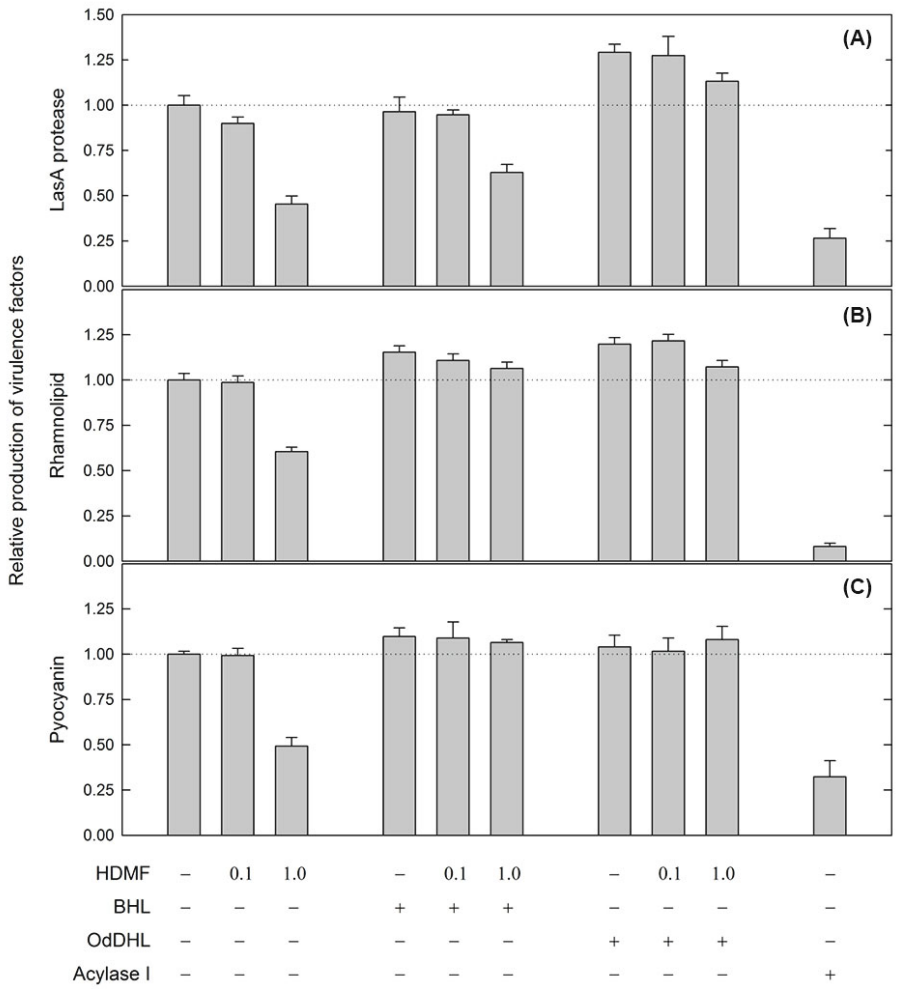

Fig. 4. The relative production of virulence factors by *P. aeruginosa* in the presence of HDMF, BHL, and OdDHL. (A) LasA protease activity, (B) rhamnolipid production, and (C) pyocyanin production. An acylase I-treated culture was used as a negative control. The dotted line denotes the relative production of each virulence factor in the absence of both HDMF and the external AHL molecule (= 1.0).

duction and reduced the inhibitory effect of 1.0 μM HDMF (7.8% reduction in inhibition compared to –HDMF/+BHL). However, LasA protease activity was not significantly enhanced by the addition of 10 μM BHL, although the inhibitory effect was reduced from 54.6 (–BHL) to 34.9% (+BHL) at 1.0 μM HDMF. The addition of either 10 μM BHL or OdDHL resulted in a complete rescue of HDMF-induced inhibition of pyocyanin production, and no significant difference in pyocyanin level was observed among the HDMF cultures and controls (Fig. 4C). The observed rescue from the effects of HDMF by increasing the concentration of QS-inducing signals (BHL and OdDHL) clearly suggests that HDMF interferes with QS through reversible competition for the binding sites of BHL and OdDHL (Manefield *et al.*, 1999).

Discussion

A number of studies investigating the interference effect of furanone derivatives on AHL-mediated QS and biofilm formation have been published (Hentzer *et al.*, 2002; Martinelli *et al.*, 2004; Kim *et al.*, 2008; Shetye *et al.*, 2013) since the anti-QS activity of halogenated furanones from the marine alga *D. pulchra* was first reported (Manefield *et al.*, 1999). As natural furanones are considered to have little or limited effect on the QS system of *P. aeruginosa* (known as the best model microorganism for screening QSI compounds), natural furanones have been chemically modified to enhance their QSI activity (Hentzer *et al.*, 2003; Martinelli *et al.*, 2004; Janssens *et al.*, 2008; Kim *et al.*, 2008). However, many of the synthetic furanones and naturally halogenated furanones

Table 3. The Effects of HDMF on AHL production
The diameter of the induced color zone was converted to the concentration of BHL or OdDHL. [a,b] *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 were used as biosensors for BHL and OdDHL, respectively.

HDMF (μM)	BHL[a]			OdDHL[b]		
	0.0	0.1	1.0	0.0	0.1	1.0
Induced zone (diameter, mm)	39.0 ± 1.0	32.0 ± 0.5	22.0 ± 0.5	41.0 ± 1.0	38.0 ± 0.5	32.0 ± 0.5
Estimated concentration (μM)	0.64	0.29	0.09	2.76	1.23	0.20

have been found to be too reactive and toxic to be applied to humans and other higher organisms, even at very low concentrations (Hentzer and Givskov, 2003; Rasch *et al.*, 2004; Khan *et al.*, 2009). To address this issue, we decided to test HDMF (also known as furaneol or strawberry furanone), a food additive natural furanone compound that is safe to consume. HDMF has antimicrobial activity against various bacteria and fungi at high concentrations (156.2–937.2 μM). No hemolytic activity against human erythrocytes was observed even at concentrations as high as 624.8 μM, which is a critical factor in practical applications (Sung *et al.*, 2007).

To ensure that HDMF influences the QS system directly (rather than indirectly by causing growth inhibition), we conducted a QSI test using HDMF at concentrations below 1.0 μM, at which little effect on growth was observed (Fig. 1). HDMF significantly reduced the production of QS signal molecules (BHL and OdDHL) when PAO1 was grown in the presence of 0.1 μM HDMF (54.7 and 55.4%, respectively) and 1.0 μM HDMF (85.9 and 92.8%, respectively) (Table 3). Shobharani and Agrawal (2010) showed that 2(5H)-furanone (500 μM) reduced the production of butyryl homoserine lactone and hexanoyl homoserine lactone by 40.0 and 31.8%, respectively, in a *Pseudomonas* strain isolated from spoiled fermented milk. Adonizio *et al.* (2008) also reported a reduction in OdDHL (5.7–45.8%) and BHL (3.5–40.7%) production when PAO1 was cultured in the presence of aqueous extracts (1 g/L) from six plants that exhibited QSI activity. Unfortunately, it was not possible to quantitatively compare the inhibitory activity of HDMF with those of the extracts, as the active ingredient(s) was not specified.

After verifying that the production of BHL and OdDHL was reduced in the presence of HDMF, the relationship between the QS system and biofilm formation in PAO1 was examined. HDMF (1.0 μM) caused a 42.6 and 84.8% reduction in biofilm formation after 24 and 48 h of culture, respectively, compared with the control (no HDMF). These results are comparable to those of Hentzer *et al.* (2002), who reported the production of flat and undifferentiated biofilms that eventually detached in the presence of brominated furanone 56 (28.5 μM). Both results may be explained by the findings of Davies *et al.* (1998), who confirmed that abnormal biofilm formation in PAO1 was due to the absence of OdDHL (the exclusive signal for the *las* QS system) and that the biofilm formed in the absence of OdDHL was much more easily detached from the surface and dispersed. In this study, at 1.0 μM HDMF a greater than 90% reduction in OdDHL production seemed to critically influence the reduction in biofilm formation at 0–24 h, as well as increase detachment between 24 and 48 h.

Swarming is described as a social phenomenon involving the coordinated and rapid movement of bacteria across a semisolid surface as well as an important factor in biofilm development in *P. aeruginosa* (Daniels *et al.*, 2004; Overhage *et al.*, 2007). It has been shown that swarming of *P. aeruginosa* is dependent on the presence of rhamnolipids whose production is regulated by the *rhl* QS system and partly by the *las* system (Köhler *et al.*, 2000; Daniels *et al.*, 2004; Caiazza *et al.*, 2005; Shrout *et al.*, 2006). We observed a more substantial inhibitory effect of HDMF on swarming motility than on swimming or twitching (Table 1), and a less production of rhamnolipid in the presence of 1.0 μM HDMF (Table 2). These results coincide with each other and strongly suggest that HDMF mainly exerts its action on the QS of PAO1.

With respect to the inhibition of biofilm formation, HDMF was found to be highly effective even at low concentrations in comparison to other studies. A 42 and 30% reduction in PAO1 biofilm was observed after 24 h of culture with eugenol (clove extract, 400 μM) and catechin (bark extract, 4 mM), respectively (Vandeputte *et al.*, 2010; Zhou *et al.*, 2013). Among furanone derivatives, 2(5H)-furanone (7.8 mM) caused a 32% reduction in biofilm production by an *Aeromonas hydrophila* strain (Ponnusamy *et al.*, 2010) and six brominated furanones (10–100 μM) caused a 50% reduction in biofilm production by *Salmonella enterica* serovar Typhimurium (Janssens *et al.*, 2008). However, the brominated furanones BF4 (100 μM) and BF10 (50 μM) did not cause any significant change in biofilm formation by PAO1 (Shetye *et al.*, 2013). Many studies investigating the inhibitory effect of furanone derivatives have focused on qualitative changes in PAO1 biofilm morphology, the reduction in thickness, and gene expression (Hentzer *et al.*, 2002, 2003; Rasmussen *et al.*, 2005); hence, there was limited opportunity to quantitatively compare the results of this study with those of others.

When screening for compounds having strong QSI activity, in addition to determining the reduction in QS signal production and biofilm formation, the evaluation of a compound's inhibitory effect on the expression of PAO1 virulence factors has been widely applied. In *P. aeruginosa*, *lasI*, and *rhlI* encode OdDHL synthetase and BHL synthetase, respectively, and the detection of OdDHL and BHL by LasR and RhlR induces the expression of virulence factors by the hierarchically organized *las*, *rhl*, and *pqs* QS systems (Schaadt *et al.*, 2013). As representative virulence factors, whose production is regulated by the *las*, *rhl*, and *pqs* systems, LasA protease, rhamnolipid, and pyocyanin were selected, respectively (Schaadt *et al.*, 2013), and the inhibitory effect of HDMF on these virulence factors was investigated.

As presented in Table 2, the production of the three virulence factors was clearly reduced (40.9–53.8%) at 1.0 μM HDMF. These results indicate that HDMF interfered with all three QS systems of *P. aeruginosa*. Catechin (2–4 mM) mediated comparable inhibition of all three QS systems of *P. aeruginosa*, *las* (30% for LasB elastase), *rhl* (10% for rhamnolipid), and *pqs* (50% for pyocyanin) (Vandeputte *et al.*, 2010). However, a single QSI substance may not always interfere with all three of the QS systems in *P. aeruginosa*. Eugenol (at 50–400 μM) showed inhibitory activity toward *las* (32–46% for LasB elastase) and *pqs* (56% for pyocyanin), whereas it had no effect on the *rhl* system (Zhou *et al.*, 2013). Adonizio *et al.* (2008) also reported that aqueous extracts (1 g/L) of the South Florida plant *Chamaesyce hypericifolia* caused a 49.3 and 37.6% reduction in LasA and pyoverdin, respectively, whereas it caused a 380% increase in LasB activity. Zhao *et al.* (2013) reported that the expression of LasA protease (68.9% decrease), LasB elastase (65.6% decrease), and pyocyanin (76.5% decrease) was suppressed by 2.5 g/L Yunnan Baiyao, a famous Chinese herbal medicine. Among brominated furanones, BF15 and BF8 (200 μM) inhibited *las* (42.6 and 43.5% against elastase B, respectively), whereas

rhamnolipid production was increased (Shetye *et al.*, 2013). Furanone 56 (17.1 μM) and furanone C-30 (10 μM) decreased LasB elastase by 52.4% and 73.9%, respectively (Hentzer *et al.*, 2002, 2003), and 2(5H)-furanone (300 μM) decreased rhamnolipid production by 73.8% (Shobharani and Agrawal, 2010).

Previous studies have suggested that the anti-QS activity of AHL analogs (e.g., furanones) may be attributed to competition with AHL molecules in binding to the AHL-binding site, but failing to induce QS-regulated gene expression (Manefield *et al.*, 1999; Teplitski *et al.*, 2000; Hentzer *et al.*, 2002). The QSI mechanism of HDMF in *P. aeruginosa* appears to be reversible competition, which may be overcome by increasing the concentration of AHLs (OdDHL and BHL) to increase the chance of AHL binding to its binding sites (LasR and RhlR) as shown in Fig. 4. The exogenous addition of either BHL or OdDHL at 10 μM almost negated the inhibitory effect of HDMF (1.0 μM) on the production of rhamnolipid and pyocyanin by PAO1. However, the addition of BHL did not increase LasA protease production or rescue the inhibition caused by HDMF. These results were attributed to the fact that the *las* system responds almost exclusively to OdDHL (Schaadt *et al.*, 2013). Hentzer *et al.* (2002) also observed that the exogenous addition of BHL (3 μM) did not induce LasB elastase expression in *P. aeruginosa* PAO-JP2. In this study, the presence of 1.0 μM HDMF caused a decrease in the production of three virulence factors by 40.9–53.8% (Fig. 4). The stoichiometric HDMF:BHL and HDMF:OdDHL ratios were 11:1 and 5:1, respectively, calculated based on the estimated concentrations of BHL and OdDHL at 1.0 μM HDMF (Table 3). Hentzer *et al.* (2002) proposed that a furanone 56:OdDHL ratio of 400:1 would cause competitive interference in *P. aeruginosa* PAO-JP2. Such a high ratio of QSI:QS signal molecules means that the signal molecule has much higher affinity for its binding site compared to that of QSI molecule; hence, it could mitigate against successful inhibition of QS activity unless the QSI compound was applied near its toxic concentration. Although HDMF is a natural and non-halogenated furanone, the QS of *P. aeruginosa* PAO1 was inhibited at relatively low concentrations of HDMF compared with the concentration ranges of QSI furanones tested in other studies: furanone 56 (brominated) at 17.5–28.5 μM (Hentzer *et al.*, 2002), furanone C-30 (brominated) at 1–10 μM (Hentzer *et al.*, 2003), BF10 and BF4 (brominated) at 50–200 μM (Shetye *et al.*, 2013), Fur-1–Fur-12 (brominated) at 10–100 μM (Janssens *et al.*, 2008), 2(5H)-furanone at 0.1–7.8 mM (Ponnusamy *et al.*, 2010; Shobharani and Agrawal, 2010). The effectiveness of HDMF at a relatively low concentration implies that HDMF has strong affinity for the AHL binding site in PAO1 QS systems and can successfully interfere with QS through competition with signaling molecules.

Although we did not explore the QSI potential of HDMF against PAO1 at the gene expression level, the results of the present study clearly demonstrate a significant reduction in QS signal molecule levels, QS-regulated production of virulence factors, and biofilm formation by *P. aeruginosa* in the presence of HDMF without any growth inhibition. To our knowledge, there have been no reports to date of the inhibition of bacterial QS by HDMF. For the first time, we report the biofilm-controlling effect and QSI potential of HDMF against *las* and *rhl* QS systems in *P. aeruginosa* PAO1.

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