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ORIGINAL ARTICLE

Evidence for the dynamics of Acyl homoserine lactone and AHL-producing bacteria during subtidal biofilm formation

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The quorum sensing signals—acyl homoserine lactones (AHLs) were directly detected in 1-9-day-old subtidal biofilms developed in a coastal fish farm by using AHL reporter strains and gas chromatography–mass spectrometry. Both methods showed that the AHL molecules and/or AHL-producing bacterial community were dynamic during biofilm development, with dominant AHLs changed from short-chain to long-chain AHLs. Terminal restriction fragment length polymorphism analysis of the bacterial 16S rRNA genes derived from subtidal biofilms of different ages was compared to that of the 21 AHL-producing bacteria isolated from the same batch of subtidal biofilms. All terminal restriction fragments (TRFs) generated from AHL-producing bacteria matched with the dominant TRFs derived from the biofilm bacterial community samples. Particularly, the TRFs of all AHL-producing *Vibrio* spp. matched with the TRFs that were dominant only in 1-day-old biofilm, suggesting that AHL-producing vibrios were one of the pioneer groups during subtidal biofilm formation. We reported here for the first time the dynamics of AHLs and AHL-producing bacteria during the formation of a subtidal biofilm.
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Introduction

Surfaces submerged in seawater are readily colonized by marine microorganisms including bacteria, diatoms, protozoa, fungi and so on. The assemblage of these attached microorganisms on a surface is referred to as a marine biofilm (Cooksey and Wigglesworth-Cooksey, 1995; Costerton *et al.*, 1995). Marine biofilms play important roles in various biological processes, such as the biofouling development, nitrogen fixation, sulfate reduction and so on, therefore are important topics in marine ecological study (Costerton *et al.*, 1995; Qian, 1999; Decho, 2000; Huang *et al.*, 2007b).

Bacteria are dominant and active members of marine biofilms. In laboratory bacterial biofilms are inextricably linked with bacterial quorum sensing (QS), the density-dependent cell–cell communication (Parsek and Greenberg, 2005). QS allows bacteria to alter their behavior on a population-wide scale in response to changes in the number and/or species present in a community (Waters and Bassler, 2005). Several phenotypes beneficial for bacterial surface colonization, including motility, exopolysaccharide production, biofilm formation and toxin production, are often regulated by QS (Davies *et al.*, 1998; Stoodley *et al.*, 2002; Parsek and Greenberg, 2005; Waters and Bassler, 2005).

N-acyl homoserine lactones (AHLs) are the best-studied QS signals among Gram-negative bacteria. AHL-based QS system was firstly discovered from marine *Vibrio fischeri* (Milton, 2006). Recently, AHL-producing bacteria were isolated from 'marine snow' (Gram *et al.*, 2002), marine sponges (Taylor *et al.*, 2004; Mohamed *et al.*, 2008), marine water column, eukaryotic algae (Wagner-Dobler *et al.*, 2005) and marine subtidal biofilms (Huang *et al.*, 2007a), suggesting the potential impacts of AHLs in marine bacterial communities. It was proposed that bacteria might use QS as a weapon to compete for survival or to dominate in a natural niche (Waters and Bassler, 2005; Defoirdt *et al.*, 2008). Indeed, in

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the laboratory, AHL was shown to mediate the interaction between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures (An *et al*., 2006), whereas AHL-QS blockers and the presence of AHLs were found to change bacterial community composition and function in natural environments and activated sludge (Valle *et al*., 2004; McLean *et al*., 2005; Dobretsov *et al*., 2007).

Marine natural biofilms are unique ecological niches where multiple species of bacteria attach and grow on a surface in a dynamic process (Costerton *et al*., 1995; Decho, 2000). Although molecular mechanisms of AHL-regulated biofilm formation had been extensively studied in the laboratory (Davies *et al*., 1998; Parsek and Greenberg, 2005; Waters and Bassler, 2005); little is known about the ecological roles of AHLs in natural biofilm formation (Manefield and Turner, 2002; Parsek and Greenberg, 2005). In our parallel study, 21 AHL-producing bacteria were isolated from subtidal biofilms of different ages (Huang *et al*., 2008). In this study, we provided evidence that bacterial AHL signals were prevalent and variable *in situ* in subtidal biofilms; and the composition of AHL-producing bacteria was dynamics during biofilm development.

Materials and methods

Development of subtidal biofilms

Subtidal biofilms were developed during 12th–21st October 2006 at Yung Shue Au fish farm, Hong Kong (22° 25′ 35N, 114° 16′ 46E). During the sampling period, the weather was stable and the average water parameters were: temperature 27.4 °C, salinity 31.7 p.p.t., dissolved oxygen 6.7 mg l^{-1}, and the pH value 8.0 (Hong Kong Environmental Protection Department).

Polystyrene Petri dishes for different experimental purposes were held in fishnets and submerged 2 m below the seawater level. Petri dishes were retrieved from the field after 1, 3, 5, 7 and 9 days of submersion and transported immediately back to the laboratory in a heat-proof container filled with *in situ* seawater.

Detection of AHLs in subtidal biofilms by AHL reporter strain

The AHL reporter strains *A. tumefaciens* strain A136 (pCF218) (pCF372) and *Chromobacterium violaceum* strain CV026 (obtained from Prof. McLean, Texas State University) were used to detect AHLs in subtidal biofilms. The strain A136 produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in response to a wide range of AHLs (McLean *et al*., 1997), whereas the strain CV026 produces a purple pigment when induced by short- to medium-chain AHLs, such as C4-, C6-, C8-HSL and 3-oxo-C6-, C8-HSL (McClean *et al*., 1997).

The AHL reporter strain was streaked on the Luria Broth agar plate (diameter = 90 mm, Sterilin, UK). For A136 bioassay, 50 μl X-Gal (20 mg ml^{-1}) were spread on each plate before bioassay was performed. Then the smaller Petri dish (diameter = 50 mm Falcon #1006, USA) with subtidal biofilm developed on it was placed approximately 1 cm apart from the streak, with biofilms directly in contact with agar (Figure 1). Five replicates were performed for each day biofilm. Experimental plates were then incubated at 28 °C for 48 h. If AHLs are present, they diffuse through the agar and activate the coloration of the reporter strains. Assays with *A. tumefaciens* A136 vs *A. tumefaciens* KYC6 and *C. violaceum* CV026 vs *C. violaceum* ATCC 31532 served as positive controls. Assays with the reporter strain vs itself and vs clean dish served as negative controls.

Profiling of AHLs in subtidal biofilms by gas chromatography—mass spectrometry

On each sampling day, subtidal biofilms were harvested by swabbing both sides of 100 Petri dishes (diameter = 90 mm) with sterile cotton buds. Biofilms were extracted three times with 300 ml of ethyl acetate (EA) containing 0.2% (v/v) glacial acetic acid. The EA extracts were combined and the solvent was removed by evaporation under reduced pressure; the extracts were freeze dried, redissolved

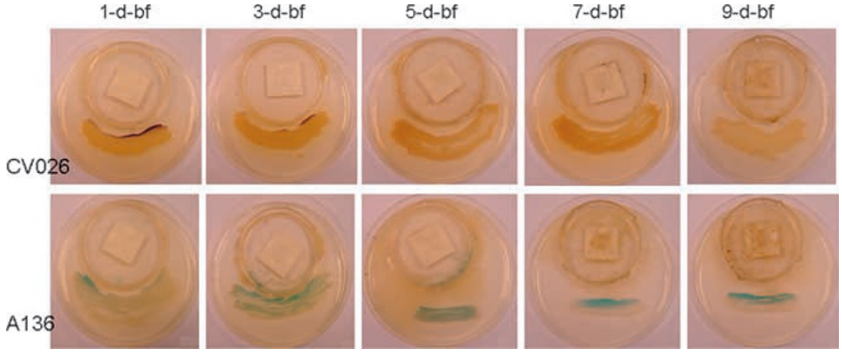

Figure 1 Detection of acylated homoserine lactones (AHLs) in subtidal biofilms of different ages (1–9-day-old biofilms, 1–9-d-bf) using the reporter strains *Chromobacterium violaceum* CV026 (fist raw panels) and *Agrobacterium tumefaciens* A136 (second raw panels). Biofilms were developed on the Falcon #1006 Petri dishes (diameter = 50 mm) and placed onto Luria Broth (LB) agar plates (diameter = 90 mm) with the reporter strains streaked 1 cm apart.

in 1 ml of dichloromethane and then subjected to gas chromatography—mass spectrometry (GC-MS) analysis.

Gas chromatography—mass spectrometry (Varian/CP-3800 and Varian/Saturn 2200) was incorporated with a relatively nonpolar capillary column (CP-Sil 8 CB-MS, 30-m length, 0.25 μm film thickness, 0.25 mm internal diameter). The injection port and the interface were held at 220 and 300 °C, respectively. The temperature was programmed from 120 to 275 °C at 10 °C per minute, followed by a 15 °C per minute ramp to 300 °C and a hold at 300 °C for 5 min with helium as the carrier gas.

AHLs in the samples were identified by comparing to standard AHLs and also to specific fragment patterns reported in (Cataldi *et al.*, 2004). Standard AHLs included *N*-butyryl-, hexanoyl-, octanoyl-, decanoyl-, dodecanoyl-, tetradecanoyl-homoserine lactone (C4-, C6-, C8-, C10-, C12-, C14-HSL) and *N*-3-oxo-butyryl-, hexanoyl-, octanoyl-, decanoyl-, dodecanoyl-homoserine lactone (3-oxo-C4-, C6-, C8-, C10-, C12-, C14-HSL), and *N*-3-OH-butyryl-, hexanoyl-, octanoyl-, decanoyl-homoserine lactone (3-OH-C4-, C6-, C8-, C10-HSL), which were purchased from the laboratory of Prof. Paul Williams, UK. AHLs with C4- and C6- side chain were grouped into short-chain AHLs. AHLs with C8- and C10- side chain were grouped into medium-chain AHLs. AHLs with C12- and C14- side chain were grouped into long-chain AHLs.

Collection and extraction of genomic DNA from biofilms and water column

On each sampling day, biofilms from five Petri dishes (diameter = 90 mm) were rinsed with artificial seawater and swabbed with sterile cotton buds to collect the bacterial community. On the first sampling day, 3 l (for three replicates) of seawater were collected at the biofilm sampling site. Seawater was filtered first through a 100-μm-pore-size polycarbonate membrane (Millipore, Bedford, MA, USA) and subsequently through a 0.22-μm-pore-size membrane (Millipore). Each 0.22-μm membrane or cotton bud was immediately frozen in 0.8 ml of extraction buffer (100 mM Tris-HCl, 100 mM Na_2–EDTA, 100 mM Na_2HPO4, 1.5 M NaCl, 1% cetyl trimethylammonium bromide; pH 8). Bacterial cells in the samples were lysed in two freeze-thaw cycles, using liquid nitrogen and a 65 °C water bath. Total DNA was extracted and purified according to the SDS-based method described in Zhou *et al.* (1996). Purified DNA was dissolved in 50 μl of sterile H_2O and kept at −20 °C until use.

Amplification of the 16S rRNA gene by PCR

The 16S rRNA genes in the DNA samples were amplified by PCR using the primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 926R-fluorescein (Fam; 5′-CCGTCAATTCCTTTRAGTTT-3′; Liu *et al.*, 1997). The latter was labeled at the 5′ end with 6-carboxy Fam. Each PCR mixture contained 3 μl of DNA sample, 1.25 U of Taq polymerase (Amersham Biosciences, Piscataway, NJ, USA), 0.25 mM of dNTPs, 0.1 μM of each primer, 1.5 mM of $MgCl_2$ and 2.5 μl of 10 × PCR buffer in a total volume of 25 μl. PCR was performed in the following thermal cycles: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min and 72 °C for 10 min. PCR products were checked on 1% agarose gel. Desired PCR products were purified using a PCR Purification Mini Kit (Watson, China) according to the manufacturer's instructions. For each sample, PCR was performed with three replicates for later on terminal restriction fragment length polymorphism (TRFLP) analysis.

Terminal restriction fragment length polymorphism

PCR products were cleaved with 10 U of the restriction enzyme *Msp*I at 37 °C for 6 h. Digested products were precipitated with three volumes of cold absolute ethanol, centrifuged (12 000 r.p.m.) at 4 °C for 20 min and washed with 100 μl of 75% cold ethanol. Purified products (10 μl) together with 0.5 μl of the internal size standard (ET550-R, Amersham Biosciences) were denatured at 95 °C for 2 min, snap cooled on ice and subjected to electrophoresis on a MegaBACE genetic analyzer (Amersham Biosciences) operated in the genotyping mode.

Analysis of TRFs in AHL-producing bacteria

A total of 21 AHL-producing bacteria were isolated from the same batch of subtidal biofilms in a parallel study (Huang *et al.*, 2008) and subjected to TRFLP analysis. Genomic DNA was extracted from pure colonies of each isolate using a bacterial genomic DNA extraction kit (TaKaRa, China), whereas the primers, restriction enzyme and PCR conditions to obtain TRFs from each isolate were the same as mentioned above.

Statistical analysis

All analyses of the TRFs generated from TRFLP analysis were performed using Genetic Profiler in the MegaBACE software package (Amersham Biosciences). For each sample, TRFs that were <50 fluorescence units in intensity, <50 bp in size, and >550 bp in size were excluded from statistical analysis to screen off background noise, to avoid pseudo-TRFs derived from primers and to avoid inaccurate size determination, respectively (Moesender *et al.*, 1999). The Bray–Curtis similarity matrix was constructed based on the total number of TRFs observed in all samples and the presence or absence of TRFs in individual samples. Agglomerative hierarchical clustering was performed by the 'group average' method to display a dendrogram

using PRIMER statistical software (Plymouth Marine Laboratory, Plymouth, UK). In all cases, the threshold level for significance was 5%.

Results

Presence of AHLs in subtidal biofilms over time

Representative results of the AHL reporter strain bioassays were shown in Figure 1. Among the five replicates, four of the 1-day biofilms and three of the 3-day biofilms could induce coloration of *C. violaceum* CV026, none of the 5-, 7- and 9-day biofilms induced coloration of *C. violaceum* CV026. Moreover, four out of five 1-day biofilms and all of the other biofilms induced a blue coloration of *A. tumefaciens* A136, although maximal induction was observed at 7- and 9-day biofilms (Figure 1). As the two reporter strains respond to different ranges of AHLs, the differential induction of the reporter strains suggested that dominant AHLs and/or AHL-producing bacterial communities changed in subtidal biofilms over time. No coloration was induced in the negative controls (that is, clean dishes or reporter strains themselves), whereas AHL overproducing strains (strain 31532 and KYC6) induced coloration of reporter strains in the positive controls.

Profiling of AHLs in biofilm extracts

Acyl homoserine lactones in the biofilm extracts were identified by comparing their gas-chromatographic retention times and mass spectra with those of the standard AHLs. Figure 2 shows the identification of C10-HSL in the 9-day biofilm extract by GC-MS analysis. AHL profiles in the biofilm extracts were summarized in Table 1. Diverse AHLs including C6∼C14-HSL and 3-oxo-C4∼C14-HSL (see Supplementary Figure 1 for example of 3-oxo-C8-HSL) were detected in biofilms of different ages. In the 1-day biofilms, long-chain AHLs such as C14-HSL and 3-oxo-C12, C14-HSL were absent, whereas in the 7- and 9-day biofilms, short-chain AHLs such as 3-oxo-C4, C6-HSL were absent.

TRFLP analysis of bacterial communities in subtidal biofilms and water column

With the combination of primers and restriction enzyme used in this study, the mean numbers of TRFs derived from biofilms of different ages ranged

Table 1 The AHL profile in subtidal biofilms of different ages developed in a coastal fish farm

AHLs	*Biofilm age*				
	1-d	*3 -d*	*5-d*	*7-d*	*9-d*
C6-HSL	+	+	+	+	+
C8-HSL	+	+	+	+	+
C10-HSL	+	+	+	+	+
C12-HSL	+	+	+	+	+
C14-HSL	−	+	+	+	+
3-oxo-C4-HSL	+	+	−	−	−
3-oxo-C6-HSL	−	+	+	−	−
3-oxo-C8-HSL	+	+	+	+	+
3-oxo-C10-HSL	+	+	+	+	+
3-oxo-C12-HSL	−	+	+	+	+
3-oxo-C14-HSL	−	+	+	+	+

Abbreviations: AHLs, acyl homoserine lactones; d, day; HSL, homoserine lactone.
+: present; −: absent.

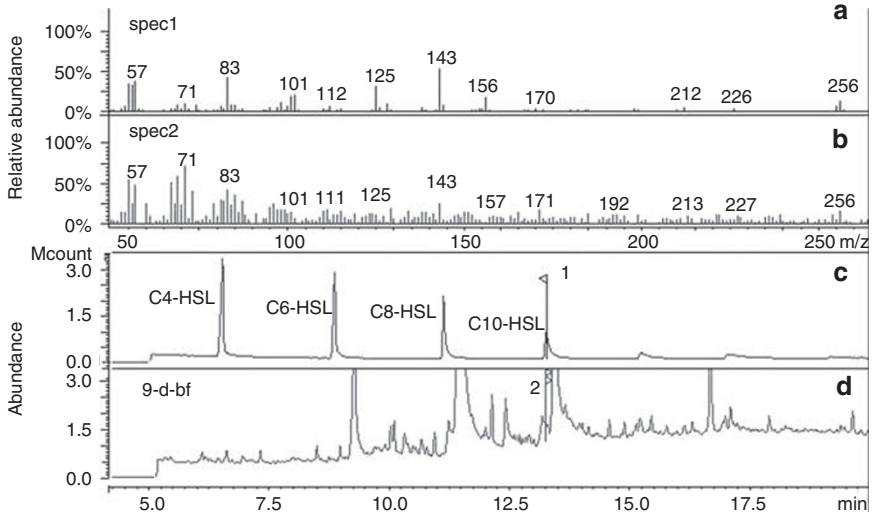

Figure 2 Identification of decanoyl-homoserine lactone (C10-HSL) in the extract of the 9-day-old biofilm. (**a**) Mass spectra of the standard C10-HSL indicated by the flag 1 in (**c**). (**b**) Mass spectra of the selected peak indicated by the flag 2 in (**d**). (**c**) Gas chromatography (GC) ion chromatogram showing the retention times of the standard AHLs. (**d**) GC ion chromatogram of the 9-day-old biofilm extract. The selected peak (indicated by flag 2) in the sample has the same retention time and similar mass spectrum as the standard C10-HSL.

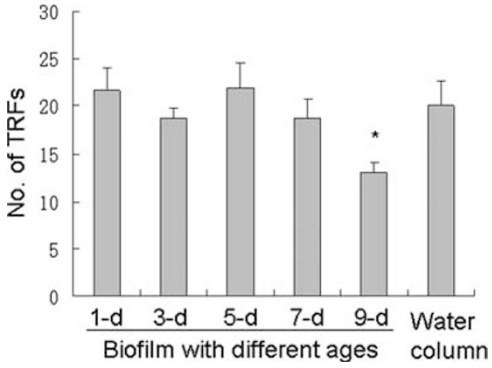

Figure 3 Number of terminal restriction fragments (TRFs) derived from *Msp*I digestion of PCR-amplified 16S rRNA gene of bacterial community DNA obtained from subtidal biofilms of different ages (1–9 day) and water column. Data plotted are mean ± s.d. of three replicates. *Data that is significantly different from the water column in *t*-test ($P<0.05$).

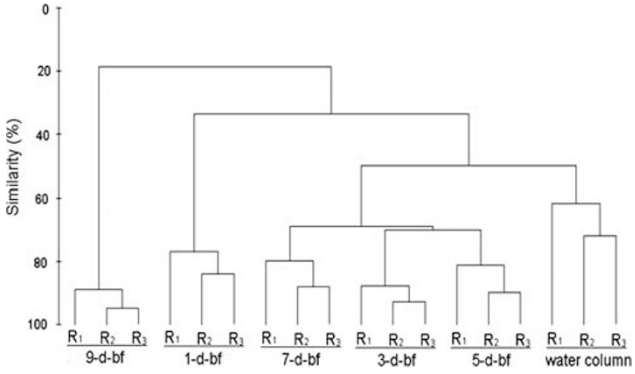

Figure 5 Dendrogram compiled from cluster analysis of presence and absence data for the terminal restriction fragment length polymorphism (TRFLP) profiles of subtidal biofilms (1–9-day-old biofilms) and water column. R: replicate samples, and the subscript number signifies replicate identity.

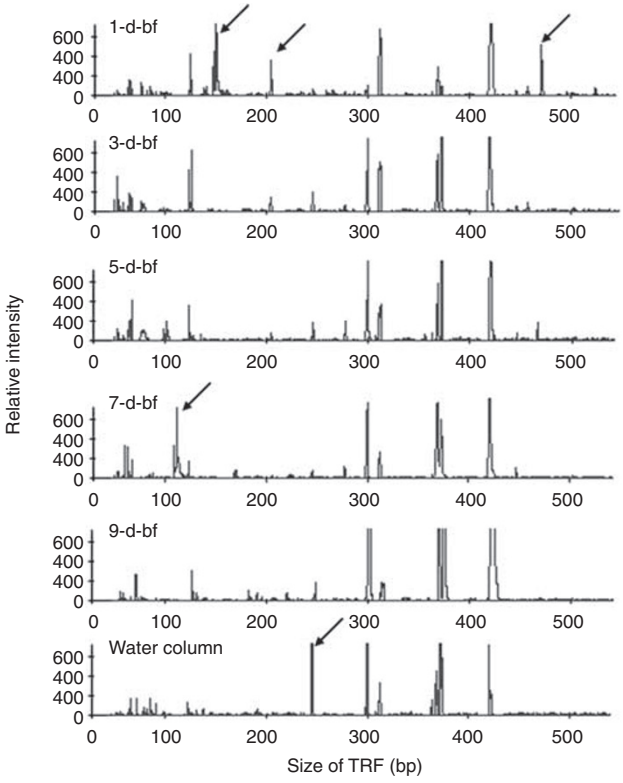

Figure 4 Electropherograms of terminal restriction fragments (TRFs) derived from *Msp*I digestion of PCR-amplified 16S rRNA gene of bacterial community DNA obtained from 1–9-day-old biofilms (1–9-d-bf) and water column. Arrows indicate unique TRFs in individual samples.

from 13 to 22, with the most abundant in the 1- and 5-day biofilms and least abundant in the 9-day biofilm (Figure 3). The average number of TRFs in water samples was 20 (Figure 3).

Electropherograms of TRFs for bacterial community derived from 1–9-day biofilms and water column were shown in Figure 4. The bacterial community composition was dynamic in biofilms and some peaks were unique compared to the water column (Figure 4). The TRFs of 312, 313 and 423 bp, were dominant in all samples. However, some TRFs were specifically dominant in certain samples, such as TRFs of 150 bp, 204 bp, 470 pb in the 1-day biofilm; 110 bp in the 7-day biofilm and 245 bp in water sample, respectively.

Cluster analysis showed that three replicates from each sample shared >75% similarity (Figure 5). The bacterial communities derived from the 3-, 5- and 7-day biofilms could be further grouped into a larger cluster sharing >60% similarity. The bacterial communities derived from the water column were distinct and shared less than 50% similarity with those derived from biofilms, indicating that the planktonic bacterial community was quite different from the attached bacterial communities. The bacterial communities derived from 9-day biofilms shared less than 20% similarity with the rest (Figure 5), suggesting that bacterial community became more distinct as the biofilm developed.

TRFs of AHL-producing bacteria

In total, 11 TRFs with sizes ranged from 71 to 423 bp were generated from the 21 AHL-producing bacteria (Table 2). With the exception of sf24, which generated two TRFs, all other bacterial isolates generated single TRFs upon *Msp*I digestion of PCR-amplified 16S rRNA gene. All of these TRFs could be matched with the TRFs derived from the biofilm bacterial community samples, and most of them contributed to the dominant peaks in the biofilms, such as TRFs of 127, 204, 300, 312, 313, 370 and 423 bp (Figure 4).

Within the sample size of this study, TRFs generated by *Vibrio* spp., the TRF of 150 and 204 bp, were unique to this group and only dominant in the 1-day biofilm community samples (Figure 4), indicating that AHL-producing vibrios attached readily to a surface and were one of the

Table 2 TRFs derived from *Msp*I digestion of PCR-amplified 16S rRNA genes obtained from individual AHL-producing bacteria isolated from subtidal biofilms

Isolate	*Access no.*[a]	*Closest match in the GenBank (Similarity)*	*Phylogenetic branch*	*TRFs (bp)*
sf11	EF587958	*Silicibacter* sp. (97%)	α-Proteobacteria	300
sf69	EF588001	*Ruegeria atlantica* (99%)	α-Proteobacteria	127
sf78	EF588009	*Ruegeria atlantica* (99%)	α-Proteobacteria	423
sf80	EF588010	*Roseobacter* sp. (99%)	α-Proteobacteria	423
sf87	EF588016	*Roseobacter* sp. (97%)	α-Proteobacteria	313
sf10	EF587957	*Vibrio* sp. (96%)	γ-Proteobacteria	204
sf27	EF587970	*Vibrio shilonii* (99%)	γ-Proteobacteria	150
sf28	EF587971	*Vibrio hepatarius* (99%)	γ-Proteobacteria	204
sf36	EF587977	*Vibrio* sp. (99%)	γ-Proteobacteria	204
sf37	EF587978	*Vibrio* sp. (99%)	γ-Proteobacteria	204
sf43	EF587982	*Vibrio* sp. (99%)	γ-Proteobacteria	204
sf41	EF587980	*Pseudoalteromonas* sp. (100%)	γ-Proteobacteria	127
sf47	EF587984	*Pseudoalteromonas luteoviolacea* (100%)	γ-Proteobacteria	312
sf51	EF587987	*Pseudoalteromonas maricaloris* (99%)	γ-Proteobacteria	312
sf52	EF587988	*Pseudoalteromonas viridis* (98%)	γ-Proteobacteria	313
sf57	EF587993	*Pseudoalteromonas viridis* (99%)	γ-Proteobacteria	423
sf85	EF588014	*Pseudoalteromonas* sp. (99%)	γ-Proteobacteria	300
sf64	EF587998	*Thalassomonas ganghwensis* (97%)	γ-Proteobacteria	313
sf24	EF587968	*Flammeovirga aprica* (97%)	Bacteroidetes	71, 312
sf75	EF588006	*Muricauda flavescens* (93%)	Bacteroidetes	125
sf76	EF588007	*Gelidibacter* sp. (94%)	Bacteroidetes	370

Abbreviation: TRF, terminal restriction fragment.
[a]Access no. of isolates were deposited in the GenBank.

pioneer groups during biofilm formation. The bacteroidetes also generated three distinguishable TRFs at 71, 125 and 375 bp. Although for AHL-producing *Pseudoalteromonas* spp. and α-proteobacteria, diverse TRFs were generated and most of the TRFs were shared in these two groups, such as TRFs of 127, 300, 313 and 423 bp.

Discussion

Dynamics of AHLs during subtidal biofilm formation

The observation that only the 1- and 3-day subtidal biofilms could induce coloration of the reporter strain CV026 was similar with that in our previous study, which was carried out at the university pier, a quite different costal marine environment (Huang *et al.*, 2007a). These consistent results suggest that dynamics of AHLs in subtidal biofilms over time may follow similar pattern in coastal marine environments. As coloration of the strain CV026 is induced only by short- to medium-chain AHLs, although inhibited by long-chain AHLs (McClean *et al.*, 1997), together with the results from the strain A136 bioassay, we propose that dominant AHLs produced by bacteria in subtidal biofilms changed from short-chain to long-chain over time. This proposal was further supported by direct profiling of AHL molecules by GC-MS analysis, which revealed that long-chain AHLs were absent in the 1-day-old biofilms, whereas short-chain AHLs were absent in the 7- and 9-day-old biofilms (Table 1). Changes in dominant AHLs could be due to the shift in AHL profiles produced by bacteria or the succession of AHL-producing bacterial community over time. Indeed, the AHL-producing bacterial community could be as dynamic as the whole bacterial community during subtidal biofilm formation. The presence of diverse AHL molecules suggests the complexity of AHL-based QS in subtidal biofilm communities.

Dynamics of AHL-producing bacteria during subtidal biofilm formation

Previous studies showed that succession of bacterial community in biofilms could happen within 3 days (Dang and Lovell, 2000), and might become stable after 6 days (Qian *et al.*, 2003). However, the dynamic pattern of a biofilm varies under different environmental conditions (Qian *et al.*, 2003; Webster and Negri, 2006). In this study, the number of TRFs in 9-day-old biofilms was less than the younger biofilms (Figure 3), whereas the major peaks in 9-day biofilms showed stronger signals than those in the younger biofilms (Figure 4), suggesting a decrease in bacterial diversity and an increase in dominance of certain bacteria in the biofilm community. Our data showed that the subtidal biofilm developed in a fish farm remained highly dynamic within 9 days of sampling period, and the bacterial community in the biofilm became more distinct from that in water column (Figure 5).

There are no specific probes or primers available to detect the AHL-producing bacteria using a PCR approach (Zhou *et al.*, 1996), thus detecting the presence of AHL-producing bacteria in their original community relies on the cultivable isolates. DNA fingerprinting methods such as TRFLP offer rapid analysis and comparison of bacterial community

structures among samples (Massol-Deya *et al.*, 1995). However, relating these community profiles to species composition is not straightforward because of the fact that different species may result in one single TRF in TRFLP analysis (Kent *et al.*, 2003). This could be complemented somehow by determination of TRFs derived from individual isolates (Lee *et al.*, 2006).

Relating the TRFs of AHL-producing isolates to biofilms community is in good agreement with the AHL reporter bioassay results and our isolating records (see Supplementary Table 1). For example, the three isolates sf28, sf41 and sf47, which were the only strains that induced coloration of CV026 (Huang *et al.*, 2008), generated TRFs of 127, 204 and 312 bp that were specifically abundant in the 1- and 3-day-old biofilms (Figure 4), which also induced coloration of CV026 (Figure 1). Moreover, AHL-producing vibrios generated TRFs of 150 and 204 bp that were only dominant in the 1-day-old biofilms but not in other biofilms (Figure 4), which coincided with our isolation records that all *Vibrio* spp. were isolated from the 1-day-old biofilms. Moreover, AHL-producing *Pseudoalteromonas* spp., α-proteobacteria and bacteroidetes were isolated throughout the sampling time; their TRFs were also dominant in TRFLP profiles over time. For example, the TRFs at 300, 312, 313, 375 and 423 bp.

Changes in the dominance of TRFs generated by AHL-producing bacteria over time were visible in TRFLP; however, other bacterial group could also contribute to these TRFs. Online analysis of possible matches to these TRFs may give us some idea about who are they (Lee *et al.*, 2006); however, the real dynamics of these groups along biofilms development requires further study and more advanced methods, such as High-Density Universal 16S rRNA Microarray Analysis method (DeSantis *et al.*, 2007).

Potential roles of AHLs in marine biofilms formation

Bacteria in natural environments tend to attach and grow on surfaces, living as biofilms (Stoodley *et al.*, 2002; Parsek and Greenberg, 2005). Interactions between members in the community are undoubtedly linked with bacterial metabolic activity (Moller *et al.*, 1998). For several species, QS has been shown to influence bacterial surface attachment and biofilm development in laboratory (Davies *et al.*, 1998; An *et al.*, 2006). Studies on natural biofilms have mainly focused on the comparison of bacterial community compositions in biofilms from contrasting environments (Qian *et al.*, 2003; Lee *et al.*, 2006), and phylogenetic identification of major bacteria constituting biofilms (Dang and Lovell, 2000). Few studies have been conducted to address the biological forces that drive the succession of bacterial community composition during biofilm development. The question remains open whether QS contribute to the dominance of certain bacteria in a multi-species natural biofilms.

In this study, all of the TRFs generated by AHL-producing bacteria belonged to the dominant peaks in the TRFLP of different biofilm samples. This is not surprising because most AHL-producing bacteria belonged to *Vibrio* spp., *Pseudoalteromonas* spp. and *Rhodobacteraceae* family (Huang *et al.*, 2008) that were frequently found to be dominant in early marine biofilm communities (Acinas *et al.*, 1999; Dang and Lovell, 2000; Lau *et al.*, 2002; Buchan *et al.*, 2005). The ability to use QS may be part of the genetic machinery that enables these bacteria to dominate in the subtidal biofilm community.

We observed that TRFs for AHL-producing *Vibrio* spp. only dominated in the TRFLP of the first day biofilm. This result suggests that vibrios in the seawater tend to attach to a surface and are one of the pioneer groups in the biofilm community. The nondominance of *Vibrio* spp. in the later time biofilms does not preclude their existence in the biofilms, but rather indicates the succession in the bacterial community composition. It had been reported that some *Vibrio* spp. employed biofilm formation to survive in the marine environments, and QS played an important role in this process (Bartlett and Azam, 2005; Milton, 2006; Mueller *et al.*, 2007). No clear succession was found in this study for other two major AHL-producing groups, the *Pseudoalteromonas* spp. and the *Rhodobacteraceae* family. Their TRFs and isolation records suggested that these bacteria were rather persistent throughout the sampling time. The importance of *Pseudoalteromonas* spp. and *Rhodobacteraceae* family in marine environments is becoming more and more significant (Holmstrom and Kjelleberg, 1999; Buchan *et al.*, 2005); the roles of QS and biofilm formation in the survival and the dominance of these two groups of bacteria also deserve more attention in the future.

As a global regulation signals for bacterial activities, AHLs may affect not only the biofilm composition, but also the consequence of biofilm activities in environment such as biofouling development, nitrogen fixation, sulfate reduction, the degradation of organic matter and, moreover, provide shelter for certain bacteria (Costerton *et al.*, 1995; Qian, 1999; Decho, 2000; Valle *et al.*, 2004; Queck *et al.*, 2006; Huang *et al.*, 2007b; Case *et al.*, 2008). For examples, the cross-domain communication by AHLs between bacteria in biofilms and spores of green alga *Ulva* indicated a direct function of AHLs in biofouling development (Joint *et al.*, 2002). Furthermore, it was reported that QS mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing (Queck *et al.*, 2006). The potential roles of AHLs in marine environments remain to be explored.

In conclusion, we reported here for the first time the dynamics of AHLs and AHL-producing bacteria during a 9-day subtidal biofilm formation. We proposed that bacterial QS signals AHLs have great

implications in the ecological functions of marine natural biofilms.

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