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ORIGINAL PAPER

Antibacterial and antilarval-settlement potential and metabolite profiles of novel sponge-associated marine bacteria

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Abstract In this study, we screened seven novel sponge-associated marine bacteria for their antibacterial and antilarval-settlement activity in order to find possible new sources of non-toxic or less toxic bioactive antifoulants. The anti-bacterial-growth activity of crude extracts of each bacterium was evaluated by the disk-diffusion assay. Extracts of four potent bacteria with high and broad spectra of antibacterial activity were further separated with solvents of different polarities (hexane and ethyl acetate). To evaluate their indirect inhibitive effect on larval settlement, we tested for their antibiofilm formation activity against two of the test bacteria (*Vibrio halioticoli* and *Loktanella hongkongensis)* inductive to *Hydroides elegans* larval settlement. About 60 and 87% of the extracts inhibited biofilm formation by *V. halioticoli* and by *L. hongkongensis* respectively. The extracts were also tested for their direct antilarval-settlement activity against the barnacle *Balanus amphitrite* and the polychaete *H. elegans*; 87% of the extracts had a strong inhibitive effect on larval settlement of both species. Extracts of two of the isolates completely inhibited larval settlement of *B. amphitrite* at 70 μg ml^{-1} and *H. elegans* at 60 μg ml^{-1}. The organic extracts of *Winogradskyella poriferorum* effectively inhibited the larval settlement of both *H. elegans* and *B. amphitrite* and the biofilm formation of the two bacterial species. The metabolites present in the active crude extracts were profiled using GC MS, and the most prevalent metabolites present in all extracts were identified. This study successfully identified potential new sources of antifouling compounds.

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Introduction

Biofouling, the colonization of submerged surfaces by living organisms such as bacteria, algae, and invertebrates [31, 37], is a widespread global phenomenon and causes serious operational problems and huge economic losses every year [5]. Man-made paints containing tributyltin (TBT) and organotin have often been used to prevent biofouling, but while these substances are undoubtedly effective, they are toxic to nontarget organisms [2]. Because of its toxicity, a worldwide ban on TBT went into effect in 2008. It is, therefore, critically important to find and develop nontoxic or less toxic antifoulants [31].

Marine organisms, from bacteria to invertebrates and plants, use chemicals to communicate and to defend themselves [9]. Chemicals that prevent colonization on living surfaces are particularly pertinent to antifouling technology and may inspire new solutions to the problem of finding safe antifoulants [9]. Although marine macro-organisms such as sponges have been shown to be good sources of bioactive compounds, including antifouling compounds, it is difficult to obtain adequate supplies of these compounds on a commercial scale. The association of bacterial symbionts with marine organisms is important to natural-product discovery and development, as these associations may obscure the origin of marine natural products thought to be produced by the host [11]. Marine sponges harbor diverse microbial communities [9, 10, 12], and recent research suggests that many bioactive compounds previously identified from sponges are in fact biosynthesized through microorganisms associated

with the sponges or are produced entirely by these microorganisms [8, 9]. These studies have prompted a new research direction in natural-product chemistry, with much recent work focusing on isolating chemical compounds from the microorganisms associated with sponges [22, 36]. In particular, sponge-associated bacteria appear to be a common source of antifouling and antimicrobial compounds [9, 36]. For example, *Alteromonas* sp. associated with the sponge *Halichondria okadai* produced ubiquinones that inhibited the settlement of barnacle larvae (*Balanus amphitrite*) [17]. Bacteria attached to the surface of marine invertebrates have been shown to contain a higher proportion of antibacterial and antifouling compounds than free-living bacteria [7]. Also, bacteria can grow rapidly and can be manipulated for the large-scale production of target metabolites, making them an ideal source of antifouling compounds. During the last 10 years, research has focused mainly on marine bacteria, fungi and microalgae, as reflected by the number of natural products described from each of these groups of microorganisms [3, 6]. Although marine microbes are a promising source of antifouling compounds, only a small number of marine bacteria have been screened so far, and only a few antifouling compounds have been successfully pooled out [29].

With the aim of finding a new source for nontoxic natural antifoulants, we examined the antifouling potential of seven marine bacterial species newly described by our laboratory (Table 1). These bacteria were isolated from marine sponges. Their host sponges have been reported to produce potentially bioactive compounds with antitumor, antifouling, and antibacterial activities [11]. Sponges such as *Lissodendoryx isodyctiyalis*, *Crambe crambe*, *Ircinia dendroides*, and *Tedania ignis* have been previously identified as potent producers of antifouling or bioactive compounds [11]. Sponges often harbor bacteria that could be the true source of their bioactive compounds, but, with the exception of *T. ignis* [32], whether these sponges contain bacterial symbionts has remained unknown. The possible existence of bacterial symbionts is especially intriguing since they could be a sustainable source of sponge-derived drugs [27]. Although in recent years there has been a rise in the number of antifouling compounds isolated from bacteria, researchers have concentrated on only specific taxa, such as *Bacillus* sp., *Streptomyces* sp., or *Pseudoalteromonas* sp., thereby leaving a vast number of possibly more potent bacteria unexplored.

Our goal was to assess the bioactivity of these novel species of sponge-associated bacteria, identify those showing significant antibacterial and antilarval settlement activity, and determine which might be the best source of bioactive compounds. In addition, we determined the metabolite profiles of the active crude extracts of these bacteria using mass spectrometry (GC MS and MALDI-TOF/TOF MS) analysis, with an attempt to identify some common peaks that may lead to bioactivities of chemical extracts of those bacteria.

Materials and methods

Bacterial cultures

Stock cultures of seven bacterial strains in 50% glycerol were obtained from the Marine Bacterial Culture Collection at the Coastal Marine Laboratory, the Hong Kong University of Science and Technology (Table 1). Aliquots of 1 ml of the stocks were individually inoculated into 10 ml of peptone–yeast extract (PY) medium containing 0.3% of yeast extract and 0.5% of peptone in 0.22 µm-filtered sea water (FSW) and incubated at 23°C to the exponential phase.

Individual bacterial isolates at exponential phase (1 day old) were propagated in 500 ml of nutrient broth and cultured at 23°C for 3 days until they reached the stationary phase. The bacterial culture was centrifuged at 3634×*g* for 25 min, and the spent culture broth was collected.

Extract preparation

For the initial screening, ethyl acetate (EA) was added to the spent culture broth at a ratio of 1:1 (v/v) and shaken vigorously for 1 h for complete extraction [30]. The solvent

Table 1 List of bacteria screened for bioactivity in this study

Name	Accession number	Source	References
Shewanella irciniae (A1)	UST040318-058	Surface of the sponge *Ircinia dendroides*	[24]
Roseivirga spongocola (A2)	UST030701-084	Associated with the sponge *Tedania ignis*	[20]
Gillisia myxillae (A3)	UST050418-085	Surface of the sponge *Myxilla incrustans*	[23]
Fabibacter halotolarans (A4)	UST030701-097	Associated with the sponge *T. ignis*	[20]
Microbulbifer mediterranus (A5)	UST040307-107	Surface of the sponge *Crambe crambe*	Unpublished
Stenothermonacter spongiae (A6)	UST030701-156	Internal of the sponge *Lissodendoryx isodictyalis*	[21]
Winogradskyella poliferorum (A7)	UST030701-295	Surface of the sponge *L. isodictyalis*	[19]

fractions were separated from the aqueous phase and dried under vacuum at 40°C to remove the solvent. Each crude extract was weighed and redissolved in a small volume of solvent and tested for bioactivity and also analyzed by using GC-MS (see below).

For further testing, the bacteria that showed a high and broad spectrum of antibacterial growth activity were mass cultured, and the spent culture media were extracted using EA as stated before to obtain crude extracts.[1] These crude extracts were gradient partitioned with hexane in order to separate compounds of different polarities [30]. The hexane (H) and EA fractions were dried under vacuum at 40°C, and the residues from each fraction were weighed. The extracts were then dissolved in 0.5% of di-methyl-sulfoxide (DMSO) and diluted with FSW to achieve the required concentrations for bioassays.

Anti-bacterial-growth assay

As an initial screening method, anti-bacterial-growth activity of the crude extracts was tested against 12 ecologically important target bacteria from the marine bacterial culture collection at the Coastal Marine Laboratory, the Hong Kong University of Science and Technology, using the standard paper-disk assays [1]. These bacteria are *Vibrio vulnificus* (UST001201-001) (B1), *V. halioticoli* (UST010723-002) (B2), *Staphylococcus haemolyticus* (UST950701-004) (B3), *S. aureus* (UST950701-005) (B4), *Pseudoalteromonas piscida* (UST010620-005) (B5), *Micrococcus luteus* (UST950701-006) (B6), *Vibrio* sp. (UST950701-007) (B7), *Ruegeria* sp. (UST010723-008) (B8), *Loktanella hongkongensis* (UST950701-009) (B9), *V. harveyi* (UST020129-010) (B10), *Rhodovulum* sp. (UST950701-012) (B11), and *Shewanella algae* (UST010723-014) (B12). The target bacteria were isolated from natural biofilm in Hong Kong waters, and seven of them showed strong inductive effect on the larval settlement of *Hydroides elegans* [17, 22]. Sterile disks (6 mm in diameter, Whatman no. 1) were impregnated with the individual crude extracts (in EA) of interest (50 μg per disk) and air dried. Test bacteria were inoculated into PY medium and incubated at 23°C until they reached an approximate density of 1.2×10^8 CFU/ml (McFarlands 1). PY agar plates (0.5% peptone, 0.3% yeast extract, 1.5% agar in FSW, 9 cm in diameter) were spread uniformly with a suspension of the target bacterial stains. The disks with extracts were then placed on the agar plate (no more than eight disks per plate). A disk loaded with 50 μg of streptomycin was used as a positive control. The agar plates were incubated at 30°C for 24 h. After the incubation, the diameter of the bacterial-growth-inhibition zone around each disk caused by the crude extracts was measured under a transmitted light source. The anti-bacterial-growth activity was categorized as weak, moderate, or high depending on the diameter of the growth inhibition zone (<1, 1–5, and >5 mm, respectively).

Anti-bacterial biofilm formation assay

The crude extracts of the four marine bacteria that showed a broad spectrum of anti-bacterial-growth activity, A3, A5, A6, and A7 (Table 1), were further partitioned into ethyl acetate and hexane fractions based on different polarities and were tested for their ability to inhibit the biofilm formation of *V. halioticoli* (B2) and *L. hongkongogensis* (B9)—the biofilm-forming bacteria that have an inductive effect on the larval settlement of *H. elegans*, a dominant fouling polychaete [17, 22]. The standard presterilized 96-well polystyrene microtiter plates were used as substrata for forming bacterial biofilms [4, 26]. Initially, a concentrated extract solution was transferred into each well except those for controls. An aliquot of 150 μl of FSW was then added to the wells to achieve a final test concentration of 50 μg ml^{-1}. After that, 50 μl of bacterial inoculum (the density was pre-adjusted to McFarlands 1–3) was added into each well ($n = 8$ per extract). The plate was incubated at 23°C for 48 h. After the allotted incubation period, in order to correlate biofilm formation with planktonic cell growth in each well, the planktonic cell fraction was transferred to new microtiter plates and the OD_{600} was measured [4]. The plate with the biofilm was rinsed with phosphate buffer saline (pH 7.2) and stained with crystal violet. The samples were immediately analyzed with a microtiter plate reader (Perkin Elmer, Vector 3) at 600 nm. Negative and positive controls were included in every plate. The positive control was 0.5% of DMSO in the FSW added to the wells; the negative controls were wells with only an appropriate volume of diluted overnight cultures of B2 and B9. The background absorbance was measured in wells containing the medium and cultures. The absorbance readings of wells with and without the extracts were similar, demonstrating that bacterial growth was not inhibited by the extracts. The inhibitive effect on biofilm formation was evaluated as the ratio of the absorbance of attached cells to that of the planktonic cells [26]. The data were square-root transformed, and the variance between the treatments and the control was analyzed using one-way analysis of variance (ANOVA). Data presented in the figures were not transformed.

Anti-larval-settlement assay

The direct anti-larval-settlement activity of the bacterial extracts was tested using cyprids of the barnacle *B. amphitrite* Darwin and competent larvae (to settle and metamorphosis) of

[1] Throughout the manuscript, "crude extract" refers to the first extracts of bacterial broth by EA. The partitioned fractions are referred to as EA and hexane.

Hydroides elegans. The larvae of *B. amphitrite* and *H. elegans* were raised to competence according to [33] and [22], respectively. The extracts of each bacterial isolate were first dissolved in a small amount of DMSO and then diluted with FSW to achieve the final concentration of 40–70 μg ml^{-1} in 0.5% of DMSO. One milliliter of the testing solution was then added into each well of a multi-well polystyrene plate (#3047, Falcon, USA). About 20 larvae were gently transferred into each well. Wells containing larvae in FSW with DMSO only were used as a negative control. In the bioassays with *H. elegans* larvae, the larvae were first treated with the phospho-di-esterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) at 10^{-4} M in FSW in order to trigger larval settlement before they were transferred into the wells. All treatments were conducted in six replicates ($n = 6$). The plate was incubated for 24–48 h at 28°C in darkness. After the incubation, the numbers of larvae that were swimming or had metamorphosed (larvae that had completed settlement and were irreversibly attached to the surface) were counted directly under a microscope, and, where appropriate, any possible toxic effects of the treatments, such as the death or paralysis of larvae, were recorded. The number of metamorphosed larvae was expressed as a percentage of the total number of larvae added into each well. No pH change was observed during or after the experiment. The differences between the treatments and the control in the larval settlement assays were compared using one-way ANOVA followed by the Dunnett test. The numbers of metamorphosed larvae were converted into percentages, and the treatments showing a 0% settlement were assigned a value of $n/4$ (n = number of larvae in each replicate well) in order to improve the analysis [38]. The data were arcsin-transformed and assumptions of ANOVA were verified using the Kolmogorov and Smirnov test before analysis. Data presented in the figures were not transformed.

MS analysis of active crude extracts

The active crude extracts were analyzed using GC-MS to profile and identify the chemical composition in the extracts. GC was performed on relatively nonpolar capillary columns (CP-Sil 8 CB-MS, 30-m length, 0.25-μm film thickness, 0.25-mm i.d.; Varian 3800).

The injection port was held at 175°C, and temperature gradients used were initially from 65 to 120°C at 10°C min^{-1} and then 120 to 300°C at 10°C min^{-1}, with a subsequent constant temperature at 300°C for 12 min. Metabolites were identified by comparison with the NIST database and standards.

In order to perform the MALDI-TOF/TOF MS analysis of bioactive peptides, the extracts of the four bacteria showing the highest anti-bacterial-growth activity were prepared as described in [25]. They were concentrated using a Sep-pak C18 cartridge (Waters) followed by a wash with Milli Q water and then peptide elution by 80% ACN. The concentrated peptides were then mixed with the matrix α-cyano-4-hydroxycinnamic acid (Sigma) and spotted on stainless steel targets for analysis using the ABI 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA) with a scan range of 900–4,000 Da. An MS/MS database search for the distinct peak clusters observed was done with Mascot and NCBInr with a mass tolerance of 100 ppm.

Results

Anti-bacterial-growth activity

Among the crude extracts of the 7 novel bacterial strains, 4 showed a high anti-bacterial activity, inhibiting at least 5 out of the 12 target bacteria (Table 2). The extract of the

Table 2 Anti-bacterial-growth activity of the crude extracts from the marine bacteria

Crude extract	Test bacteria[a]											
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
A1	–	–	–	–	–	–	–	–	–	–	–	–
A2	+	–	–	–	–	–	–	–	–	++	–	–
A3	+++	+++	–	–	–	++	–	–	–	++	+	–
A4	–	–	–	–	–	–	–	–	–	–	–	–
A5	–	++	+	++	–	+	–	–	–	++	–	–
A6	+++	+++	+	++	–	+	+	++	–	++	+	–
A7	+++	–	–	–	–	+	+	++	++	++	+	–
Control	++	+++	+	+++	++	+++	+++	+++	+++	+++	++	+++

The control was a disc embedded with 50 μg of streptomycin. Experiments were carried out in triplicate

– No activity, + weak activity (<1 mm clear zone), ++ moderate activity (1–5 mm clear zone), +++ high activity (>5 mm clear zone)

[a] Test bacteria are *Vibrio vulnificus* (B1), *V. halioticoli* (B2), *Staphylococcus haemolyticus* (B3), *S. aureus* (B4), *Pseudoalteromonas piscida* (B5), *Micrococcus luteus* (B6), *Vibrio* sp. (B7), *Ruegeria* sp. (B8), *Loktanella hongkongensis* (B9), *V. harveyi* (B10), *Rhodovulum* sp. (B11), and *Shewanella algae* (B12)

bacterium A6 showed the broadest spectrum of activity, inhibiting 9 of the 12 target bacterial strains, followed by that of A7, which inhibited 7 of the target bacteria. Of the remaining three extracts, one (A2) demonstrated very poor activity and two (A1 and A4) showed absolutely no activity; these three were therefore eliminated from further study.

Anti-bacterial biofilm forming activity

Five of the extracts significantly inhibited the biofilm formation of B2 ($F = 40.606$, $P < 0.0001$), and five extracts inhibited B9 ($F = 22.041$, $P < 0.001$) (Fig. 1). Hexane extracts of A3, A5, and A7 inhibited the biofilm formation of both bacterial species. The remaining extracts showed differing levels of activity on the biofilm formation of the two bacterial species.

Anti-larval-settlement activity

The EA and hexane H fractions of the four active marine bacteria were also evaluated for their anti-larval-settlement activity against competent larvae of *B. amphitrite* and of *H. elegans* (Fig. 2). The percentage of larvae that metamorphosed in the control wells ranged from 70 to 80%, suggesting that the larvae were competent to metamorphose. Seven of the eight tested extracts significantly inhibited the larval settlement of *B. amphitrite* at a concentration of 70 μg ml^{-1} ($F = 202.23$, $P < 0.01$). All eight extracts significantly inhibited the larval settlement of *H. elegans* ($F = 1{,}358.8$, $P < 0.001$). Four of them completely inhibited the larval settlement of both *H. elegans* and *B. amphitrite* at 60 and 70 μg ml^{-1}, respectively. In particular, the EA fraction of A6, the H fraction of A3, and both the EA and H fractions of A7 completely inhibited the larval

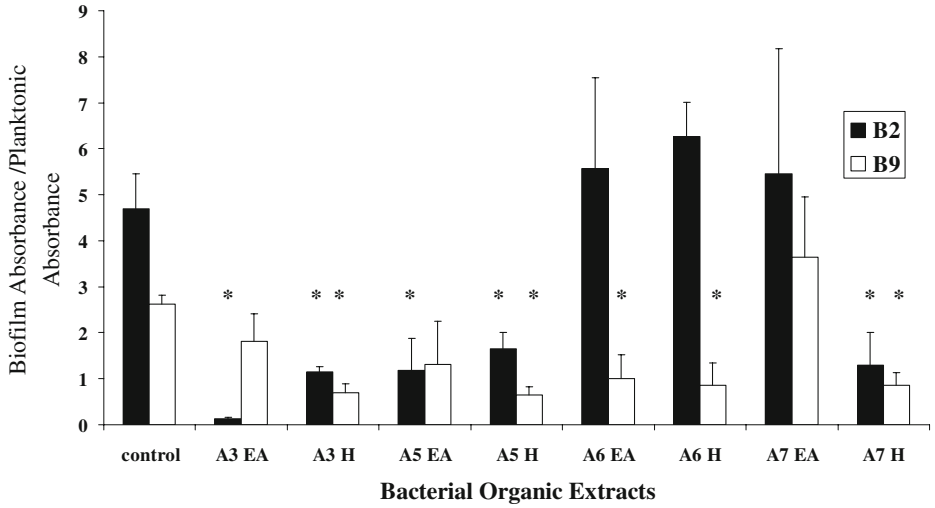

Fig. 1 Effect of bacterial organic extracts on biofilm formation of *Vibrio halioticoli* (B2) and *Loktanella hongkongenesis* (B9). Data plotted are means + SD of eight replicates. The inhibitive effect is expressed as the ratio of biofilm absorbance to planktonic absorbance at 600 nm [4]. *Asterisk* indicates a significant inhibition on biofilm formation when compared with the control (one-way ANOVA, Dunnett test, $P < 0.05$). The tested novel bacteria were A3, A5, A6, and A7 (refer to Table 1 for abbreviations). *EA* Ethyl acetate fraction, *H* hexane fraction

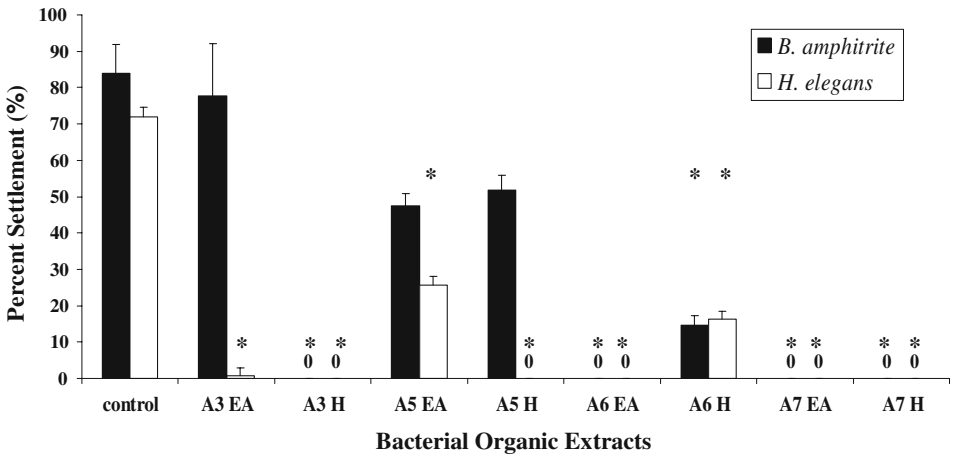

Fig. 2 Anti-larval-settlement activity of bacterial extracts against larvae of *Balanus amphitrite* and *Hydroides elegans*. Data plotted are means + SD of six replicates. *Asterisk* indicates a significant inhibition on larval settlement when compared with the control (one-way ANOVA, Dunnett test, $P < 0.05$). The tested novel bacteria were A3, A5, A6, and A7 (refer to Table 1 for abbreviations). *EA* Ethyl acetate fraction, *H* hexane fraction

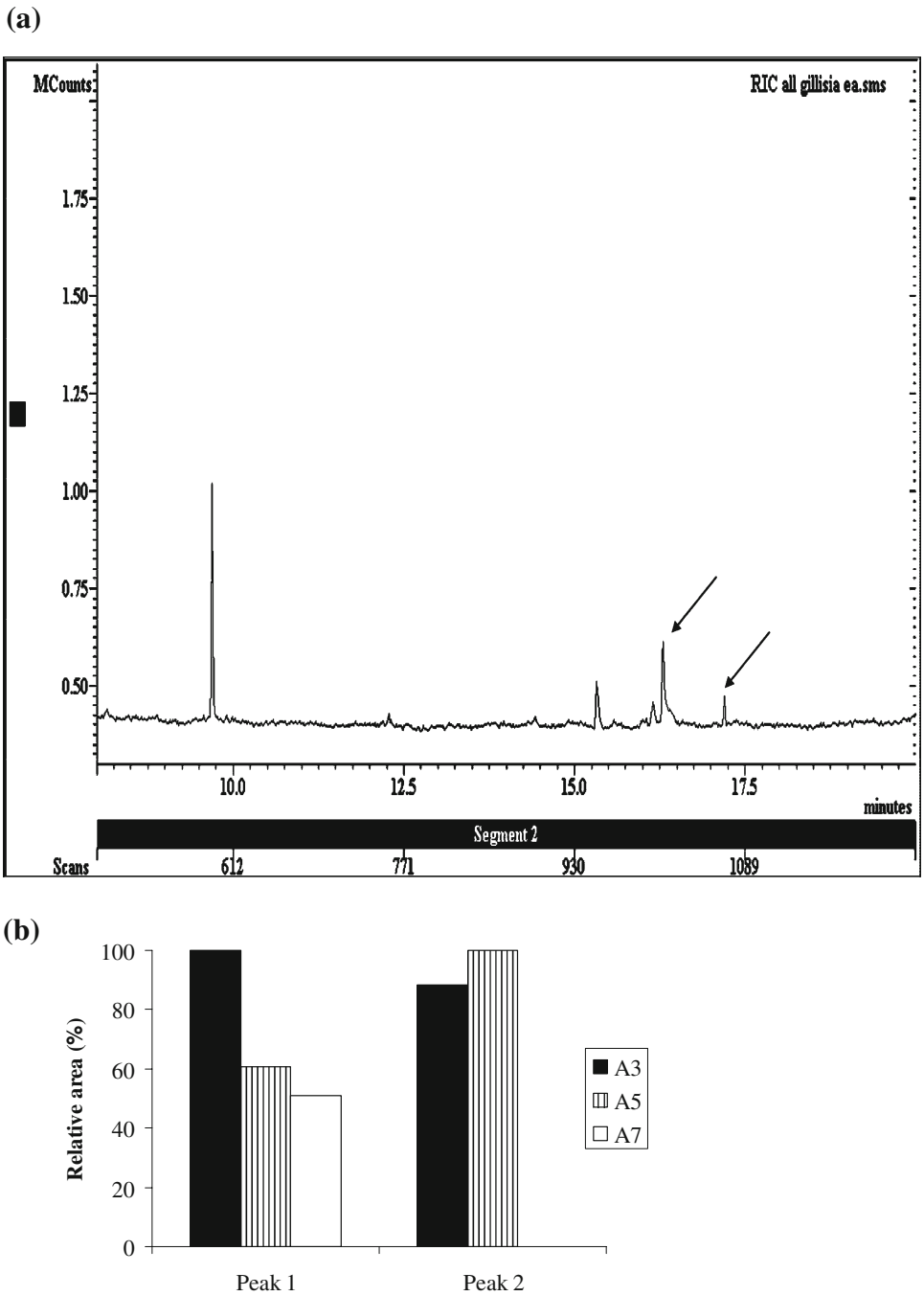

Fig. 3 **a** Gas chromatographic spectra of the crude extracts of the novel bacteria A3, A5, A6, and A7 (refer to Table 1 for abbreviations). *Arrows* show the overlapping peaks present in the different extracts. **b** Relative abundance (%) of peak 1 (R_t = 16.302) and peak 2 (R_t = 17.181) in the different crude extracts

settlement of both species. The H fraction of A3 and the EA fraction of A5 showed an inhibitive effect on larval settlement, but these extracts were found to be toxic to the larvae at these concentrations, as high levels of mortality and paralysis were observed in these treatments (data not shown).

MS analysis of bioactive crude extracts

The crude extracts of A3, A5, and A7 showed several overlaps in their GC profiles (Fig. 3a). For example, peaks at retention times of 16.302 and 17.181 min were present, in different amounts, in all three extracts (Fig. 3b). A database search for the predicted mass showed 60% similarity of the peak occurring at ~16.30 to Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl). We did not find any matches for the other peak. A library search of the other mass spectra obtained for the crude extracts showed the presence of several quorum-sensing molecules such as C10 HSL, C14 HSL, and some other organic compounds (Table 3). Several spectra had no matches in the database, suggesting the presence of some new, unexplored metabolites.

Table 3 Identity of metabolites present in the crude extracts of A3, A5, A6, and A7 identified by NIST database and AHL standards

Extract from	R_t (min)	Peak identity
A3	9.673	Benzene ethanamine, *N*(-1-methyl ethylidene)
	15.344	3-Hydroxy-1-(4-{13-[4-(3-hydroxy-3-phenylacryloyl)phenyl[tridecyl]-phenyl)-3-phenylprop-2-en-1-one
	16.31	Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl)
	17.166	*N*-3-oxo-C6-HSL
A5	9.92	Indole
	12.22	C10 HSL
	16.27	Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl)
	18.22	Acetic acid octadecyl ester
	19.859	Phenol, 2,2-methyl bis 6-(1,1-dimethyl ethyl 4-methyl)
A6	11.161	3-Hydroxy-1-4 (13-(4(3-hydroxy-3 phenyl acryloyl) phenyl) tridecyl)ph
	12.42	Butylated hydroxytoluene
	12.69	Canthaxanthin
	14.025	1H-Indene-2 butyl 5-hexyloctahydro
A7	9.22	Octadecanoic acid, {1-[(1-oxohexadecyl)oxy]methyl}-1,2-ethanediyl ester
	13.685	C 14 HSL
	14.64	C4 HSL
	15.27	N3 Oxo C10 HSL
	16.341	Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl)

R_t Retention time in minutes

Table 4 Summary of top five matched peptides with the database using Mascot search for the extracts of A3, A5, A6, and A7 analyzed by MALDI TOF/TOF MS

Extract from	Observed mass	Probable protein	Accession number
A3	999.3423	Histidine-rich metal-binding protein	GI15612385
	943.456	O antigen polymerase	GI16611786
	955.4955	Acetyl co A transferase	GI39934773
	955.4194	Betaketothiolase	GI30250194
	955.4744	Mannosyl transferase	GI49477280
	955.4731	Glutamate synthase (small subunit)	GI52211179
A5	942.489	FeS cluster-containing hydrogenase	GI42631976
	907.488	Secreted protein	GI21221157
	942.3912	Ferrochelatase	GI59712138
	942.4222	Metal ion transporter protein	GI56707740
	942.4639	Heat shock protein	GI8134495
	900.3403	NADPH quinoline oxidoreductase	GI17232542
A6	932.356	Glycosylhydrolase	GI29348435
	932.356	S Adenosyl methionine synthetase	GI56417266
	932.356	Aminopeptidase	GI42783866
	932.356	Shikimate dehydrogenase	GI57867066
	900.345	Phenol hydroxylase	GI3046914
A7	900.4417	DNA helicase II	GI45656320
	900.4417	Putative helicase	GI50402583
	984.4506	Cell-division protein FtsZ	GI38519867
	900.4244	Preprotein translocase subunit	GI41407632
	968.4683	Lipoprotein	GI21219051
	900.4417	Translation initiation factor	GI52842971

MALDI-TOF MS profiling of the EA extracts of the culture supernatant of the isolates A3, A5, A6, and A7 showed several distinct peak clusters (data not shown). A database search based on the *m/z* values by the Applied Biosystems software showed the presence of proteins involved in cell-cycle and membrane-transport proteins (Table 4). For example, the extract of A3 showed the presence of O antigen polymerases beta-ketothiolase, whereas the extract of A5 showed several unidentified proteins and some metal-binding and chelating proteins. The extract of A7 may contain helicases. Several common *m/z* values were shared amongst the four bacterial extracts, for instance 1,000.45, 1,547.638, 1,035.5 and 1,044.47, but no relevant database match was found for these peaks. The most distinct peaks shared by all extracts had no match in the database, indicating new peptides.

Discussion

In this study, the EA extracts of seven previously unstudied, sponge-associated bacteria were tested for antibacterial activity for the first time. The active extracts were further partitioned into two fractions of different polarities, the ethyl acetate fraction and the hexane fraction, in order to facilitate rapid compound isolation and identification at later stages and to determine if the activity resided in nonpolar or less polar fractions. The screening process consisted of a multi-species targeting system and included assays to evaluate anti-bacterial growth, anti-bacterial-film formation, and anti-larval-settlement activities. Recent investigations have suggested that bacteria play a critical role in the larval-settlement process of benthic marine invertebrates by producing stimulatory or inhibitory chemical signals [9, 18, 28]. Marine biofilms often induce or enhance the settlement of invertebrates [18, 28], and several studies have suggested that effectively inhibiting the biofilm formation of settlement-inducing bacteria would help protect submerged surfaces from the settlement of macrofoulers [35]. Bioactive compounds with both antibacterial and anti-larval-settlement activity may provide a better antifouling solution than those compounds that have only one type of activity [29]. The process by which bacterial extracts are screened for their potential value to marine technology, then, should follow a two-step procedure: screening first for antibacterial activity and then for anti-larval-settlement activity [35].

With this in view, the ethyl acetate extracts of the bacteria we examined were first assessed for their anti-bacterial-growth activity against 10 testing bacteria isolated from marine biofilm. Some of these test bacteria have been described as potential human pathogens or are involved in coastal-water contamination or fish and shellfish diseases; it is of great economic importance, therefore, to find organisms possessing antibacterial activity against them. The screened isolates that showed a strong antibacterial activity were further screened for their anti-biofilm-formation activity against two larval-settlement-inducing bacteria to determine whether they indirectly inhibited larval settlement. We also screened these bacteria for their direct anti-larval-settlement activity using cyprids of the barnacle *B. amphitrite* and competent larvae of the polychaete *H. elegans,* the most common biofoulers in Hong Kong [14, 15]. This is an easy and effective method to screen for antifouling compounds since it targets both bacterial biofilms and larval settlement process and also can be applied to extensive databases.

The crude extracts were analyzed using GC-MS in order to obtain a basic idea of the metabolites present in each and to compare the profiles of each extract. Several quorum-sensing signals were found in the extracts of A3, A6, and A7. We hypothesized that some of these molecules may compete with the quorum-sensing signals in the biofilm test and therefore affect biofilm formation. Surprisingly, the extracts of A3, A5, and A7 had some major peaks in common (Fig. 3a). We assume that these peaks are relevant to the potent bioactivity shown by the extracts. According to the RIC data and the GC (Fig. 3b), peaks were present at 16.302 corresponding to Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl) and at 17.181, but the corresponding mass spectra could not be identified in the database. The GC profiles of extracts of A6 showed no overlay with the other extracts.

MALDI-TOF MS has been reported as an innovative and highly efficient technique for the rapid typing of microorganisms by analysis of their secondary metabolites [34]. Hence, in our study we employed this technique to obtain fingerprints of peptides in the crude EA extracts for comparison. There were several common peak clusters among the four bacterial extracts. Prominent groups of mass peaks were observed in the mass range between 1,000 and 1,500 *m/z*. A manual comparison with the mass numbers reported previously for certain *Bacillus* spp. [34] suggested the surfactin iturin with *m/z* 1,133.498 might be present in the EA extracts of A3, A5, and A7. Also, the bacterium A7 produced a peak that could match dextranicin (at *m/z* 1,801. 745) (Fig. 3b). Dextranicin is a bacteriocin, previously reported to be produced by *Leuconostoc* spp. [13]. Several intracellular peptides—mostly the housekeeping proteins—were identified by the database owing to their abundance and the bias of the database. Multiple MS/MS steps may be necessary to unambiguously identify the peptides present in the crude extracts. Presumably these molecules were responsible for the bioactivity observed in the bioassays. However, we cannot

rule out the possibility that the presence of other natural products contributed to this bioactivity, and therefore a detailed chemical analysis is needed. Several other distinct peak clusters were found, but they could not be matched to any known bioactive peptides in the database. This may indicate the presence of certain new peptides, and further separation and purification can provide a clearer answer to this.

Among the bacterial species screened in this study, both the ethyl acetate and hexane fractions of *Winogradskyella poriferum* (A7) showed very potent anti-larval-settlement activity. The extracts of the spent culture broth of this bacterium had antibacterial activity against 7 of the 12 target bacteria, and its hexane fraction effectively inhibited biofilm formation by *Vibrio* sp. and *Loktanella* sp. The extracts inhibited the growth of a broad range of bacteria; this anti-bacterial-growth activity may provide *W. poriferorum* with a competitive edge in the marine environment, enabling it to colonize the surface of sponges and to survive as part of a cultivable bacterial community. The H fraction inhibited the biofilm formation of both B2 and B9. The EA and H fractions effectively inhibited larval settlement. Since microfouling is a prerequisite to macrofouling, the results of this indirect-inhibition assay reinforce the importance of this bacterium as a target for the further study of metabolites; this research would serve an economic purpose in helping develop nontoxic antifoulants and would also help determine ecological interactions.

Marine sponges and other invertebrates are a potential source of less toxic and more specific natural antifoulants, but obtaining a sufficient supply of antifouling compounds from them is always a difficulty [12]. So far, only a limited number of phylogenetic clades of marine bacteria and fungi have been screened for their antifouling activity [12, 16]. This study evaluated the bioactivity of some never-before-examined marine bacteria and was able to successfully and systematically pool out and identify at least 50% of these sponge-associated novel marine bacteria as having good antifouling potential. The extract of *W. poriferorum,* which showed the most significant bioactivity, will be further analyzed using bioassay-guided fractionation. The active components of these bacteria need to be fully characterized in order for us to understand their ecological significance and develop them for industrial application.

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