Proteomic and metabolomic profiles of marine Vibrio sp. 010 in response to an antifoulant challenge

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Vibrio spp. have the ability to form biofilms, which may contribute to the subsequent successful colonization by microfouling and macrofouling organisms. The effects of an antifouling compound, poly-ether B, on Vibrio sp. 010 were investigated using flow cytometry, proteomics, and metabolomics. A 2-D gel-based proteomic analysis was used to identify proteins responsive to poly-ether B treatment. The profiles of biofilm metabolites were analyzed by ultra-performance liquid chromatography-mass spectrometry. Poly-ether B caused a significant reduction in viability. The proteins affected by the treatment were related to nucleotide metabolism, the glyoxylate cycle, and stress responses. Metabolites such as tripeptides, fatty acids, and quorum-sensing molecules were regulated differentially. Down-regulation of proteins and metabolites potentially led to a loss in colonisation ability, thereby affecting the structure of the biofilm. These results suggest that the proteins and metabolites identified may serve as target molecules for potent antifouling compounds.

Keywords: poly-ether B; biofilm; Vibrio sp.; proteome; metabolome

Introduction

Biofilms are surface-associated communities of microorganisms that are enmeshed in a protective extracellular matrix (Kjelleberg & Givskov 2007). Bacterial biofilms, referred to as microfouling, initiate the colonization of new surfaces. Vibrio spp. are natural inhabitants of aquatic environments and may constitute a considerable proportion of marine biofilm communities. Biofilms of Vibrio spp. such as Vibrio sp. 010, V. haliotici, V. campbellii, and V. parahaemolyticus induce the settlement of larvae of macrofouling organisms (Lau et al. 2002; Kjelleberg & Givskov 2007). Previous studies have shown that the ability to form a biofilm is a central factor in the survival of Vibrio spp. in the aquatic ecosystem (Yildiz & Visick 2009; Whitaker & Boyd 2012). A biofilm formed by Vibrio spp. may exhibit complexity at all levels of cellular processes. Analyzing the patterns of the abundant proteins and metabolites in the biofilm allows a better understanding of the cellular metabolic networks that influence biofilm formation (Wilmes et al. 2010). For example, genomic and proteomic tools have resulted in the identification of highly expressed proteins that are involved in protein refolding and oxidative stress in a biofilm associated with acid mine drainage (Ram et al. 2005; Jiao et al. 2011). Many key proteins in the formation of biofilms have been identified, including those related to the biosynthesis of flagella, pili, and polysaccharides (Yildiz & Visick 2009). The metabolomic response of bacteria to a chemical challenge may provide crucial information about their physiological status and the ability of biofilms to survive (Boroujerdi et al. 2009).

Microbial biofilms in aquatic environments pose serious problems for the antifouling industry and aquaculture (Bragadeeswaran et al. 2011; Dobretsov & Thomason 2011; Briand et al. 2012). Natural, non-toxic antifouling compounds that exhibit settlement or growth-inhibiting properties are preferred to traditional organo-metallic biocides as antifouling agents (Omae 2003; Sipkema et al. 2005). The most promising targets for antifouling compounds are adhesive proteins, enzymes of energy production pathways, and stress response proteins (Qian et al. 2013). Small molecules such as melanogenin and uretupamine have been shown to target either a single metabolite or set of metabolites in model organisms (Kawasumi & Nghiem 2007). Previous studies have demonstrated that poly-ethers isolated from the sponge-associated bacterium Winogradskyella poriferorum inhibited the growth of Vibrio sp. 010 (Dash et al. 2011) and the settlement of barnacle cypris larvae (Dash et al. 2012).

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The simple chemical structure and broad spectrum of biological activity characteristic of poly-ethers make them potential candidates to inhibit both microfouling and macrofouling. However, the molecular mechanisms by which poly-ethers inhibit the formation of biofilms remain largely unknown. In order to understand the physiological responses of biofilms challenged by antifouling compounds, documentation of proteomic and metabolomic responses is required. The information gained may lead to the discovery of target antifoulant proteins/metabolites or molecular pathways (Han & Lee 2003). Thus, the objective of the present study was to understand the mechanism by which poly-ether B inhibits the formation of biofilms at the cellular and molecular level. The hypothesis proposes that the inhibition of biofilm formation by poly-ether B occurs through changes in the pattern of protein expression and metabolite production. To test this hypothesis, the effects of poly-ether B on biofilms of Vibrio sp. 010 were examined using cell viability assays alongside proteomic and metabolomic tools.

**Materials and methods**

**Bacterial culture**

A schematic representation of the experimental design used for this study is shown in Figure 1. Stock cultures of *Vibrio* sp. 010 (AY241433) were obtained from the Marine Bacterial Culture Collection at the Coastal Marine Laboratory of the Hong Kong University of Science and Technology. One ml aliquots of the stock were individually inoculated into 10 ml peptone/yeast extract (PY) medium containing 0.3% yeast extract and 0.5% peptone (Oxoid Ltd, Basingstoke, Hampshire, UK) in 0.22 μm filtered seawater (FSW). Cultures were incubated at 23 °C overnight to allow the bacteria to grow to exponential phase.

**Biofilm development**

Planktonic cell cultures of *Vibrio* sp. 010 were grown overnight at 23 °C in 100 ml of PY medium with agitation in a Cenviron incubator (Shelton Manufacturing Inc. Cornelius, OR, USA). The cultures were harvested from the broth by centrifugation (3500 RCF, 10 min) and washed with autoclaved filtered seawater (AFSW). Cells with an approximate density of $1.2 \times 10^8$ CFU ml$^{-1}$ (optical density (OD) of 0.6 at 600 nm) were resuspended in 5 ml of AFSW (Lau et al. 2003). The cells were then either exposed to 40 μg ml$^{-1}$ of poly-ether B (treatment) or left unexposed (control). Suspensions of control and treated samples were added to a glass Petri dish (Steriplan; 50 mm diameter, Sterilin Ltd, Newport, UK) and incubated at 24 °C under static conditions for 6, 12 and 24 h to allow the development of a bacterial monolayer.

**Determination of the effective concentration of poly-ether B on the formation of biofilms**

The isolation and characterization of poly-ether B (Figure S1) [Supplementary material is available via a multimedia link on the online article webpage] from the sponge-associated marine bacterium *W. poriferorum* is described in Dash et al. (2011). In the present study, the inhibitory effect of poly-ether B on the formation of biofilms at both the cellular and molecular levels is evaluated. To test the ability of poly-ether to inhibit *Vibrio* spp. biofilm formation and to measure overall cell numbers, a crystal violet binding assay was performed (Bhaduri et al. 1987; Dash et al. 2009). A time course (30 min–36 h) for cellular attachment at different doses of poly-ether B was performed at concentrations ranging from 2 to 200 μg ml$^{-1}$. The assay was performed under static conditions in 96-well polyvinyl chloride microtiter plates (BD Biosciences, Franklin Lakes, NJ, USA) using

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**Figure 1.** Schematic representation of the experimental design for the cell viability, proteomics, and metabolomics studies.
0.1% dimethyl sulfoxide in FSW as a control. The cells were stained with crystal violet (Sigma-Aldrich, Steinheim, Germany). The background absorbance was measured in wells containing the medium and diluents and subtracted for data analysis. To correlate the formation of biofilms with planktonic cells, the OD_{600} of attached cells was measured separately and the absorbance measurements obtained were related to the absorbance of the planktonic cells (O’Toole & Kolter 1998; Burmolle et al. 2006). The minimum biofilm eliminating/eradicating concentration is the lowest concentration that prevented the growth of the Vibrio sp. in comparison with the control. Where necessary, the data were square-root transformed to meet the assumption of normality and further analyzed using two-way analysis of variance (ANOVA) (p<0.001) followed by the Bonferroni multiple comparison test (Zar 1996).

Analysis of the biofilm structure using confocal fluorescence

Treated and control biofilms were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 2 h. The exopolymeric substances were stained with fluorescein isothiocyanate concanavalin A (FITC-Con A; Sigma, St Louis, MO, USA) at a concentration of 1 mg ml⁻¹ as described by Huang et al. (2007). The samples were visualized under 40× objective lens attached to a Zeiss inverted fluorescence microscope equipped with an argon laser (Axiovert 25, Carl Zeiss, Jena, Germany). The blue and green fluorescence was detected by excitation at 488 nm, and emission was collected with a 515–565 nm bandpass filter. Images of the biofilms were generated using Zen 2009 AxioVision software (Carl Zeiss, Jena, Germany). Three replicate biofilms were formed for each treatment. The number of image stacks of each biofilm depended on its total thickness. Sixty seven image stacks from the control and 13 from the treated biofilm were collected and quantified using the program COMSTAT. Briefly, a series of horizontal (x-y) optical sections were taken throughout the length of the biofilm. Z-stack axis measurements (the control biofilm: ~67 μm and the treated biofilm ~20 μm) were collected. The total intensity of the biomass of the biofilm was obtained by summing the intensity of all the x-y planes in the z-stack.

Fluorescent differential cell viability staining and flow cytometry

A two-color fluorescence cell viability assay followed by flow cytometry analysis was used for simultaneous determination of live/dead cells following treatment with poly-ether B. To obtain sufficient cell biomass for extraction in the treated samples and to evaluate the overall effect of the compound, both planktonic and surface-attached cells treated with 40 μg ml⁻¹ for 30 min after 24 h time intervals were analyzed. Attached cells were removed from the surface of the glass dish, centrifuged, and resuspended in FSW. The cells were then fixed in 100% ice cold ethanol for 1 h at 4 °C to prevent cell aggregation and were subsequently stored at −20 °C. Planktonic and surface-attached cells were thawed, centrifuged at 2700 × g for 5 min, washed twice with PBS (pH 7.2) and then diluted (×1000) in PBS. Another batch of cells (not fixed) was washed, resuspended, and diluted ×1000 in PBS. These cells were stained with propidium iodide (PI; 5 μg ml⁻¹) for 30 min followed by fluorescein isothiocyanate (FITC; 5 μg ml⁻¹) (Sigma, St Louis, MO, USA) as described by Miller and Quarles (1990). Acquisition rates of 25,000 events s⁻¹ pertaining to exactly 1 ml of cells were recorded using a FACS Aria II flow cytometer equipped with a blue (488 nm) argon laser (BD Biosciences, San Jose, CA, USA). The flow cytometer was optimized using the cytometer setup and tracking beads. The bacterial population was positioned such that it was entirely within the scale of a forward scatter (FSC) vs side scatter (SSC) plot. The photomultiplier tube voltage settings for FITC and PI were adjusted using unstained cells (negative control) so that the single population fell in selected channels. The bacterial population was analyzed using a combination of FSC, SSC, FITC, and PI plots with respect to the control time-matched, untreated cells. Dead and live cell populations were differentiated using FITC vs PI staining.

Cellular protein extraction, two-dimensional electrophoresis (2-DE) and image analysis

Planktonic and biofilm cells were cultivated according to the protocol described above for biofilm development. The bacterial cells were exposed to either 40 μg ml⁻¹ of poly-ether B for 24 h (treated) or left unexposed (control). Following incubation for 24 h, attached and planktonic cells were pooled, centrifuged at 2700 × g for 25 min, and washed twice with PBS. The cells were resuspended in lysis buffer containing 10 mM Tris-HCl pH 8.0, 5 mM ethylenedinitrilotetraacetic acid, and 1 mM phenylmethylsulfonylfluoride. The proteins were extracted according to Lee et al. (2006) and quantified using a modified Bradford assay (Ramagli & Rodriguez 1985). This modified assay quantifies protein samples containing any reagents, such as urea, carrier ampholytes, non-ionic detergents, and thiol compounds that are incompatible with the Lowry, Biuret, or Bradford reagent. The protein samples were acidified prior to dilution, and protein concentrations within a range of 0.5–10 μg ml⁻¹ were determined. Two hundred and fifty mg of protein (final volume 300 μl) were loaded onto immobilized pH gradient (IPG) strips with a pI range of 4–7 (Bio-Rad, Hercules, CA, USA). The strips were passively rehydrated for 30 min and then actively rehydrated for 15 h. Isoelectric
focusing (IEF) was performed using a Protean IEF cell (Bio-Rad, Hercules, CA, USA) for a total of 60 kVh (Chandramouli et al. 2012). After focusing, the strips were reduced and alkylated using equilibration buffers containing dithiothreitol and iodoacetamide, respectively. The reduced and alkylated strips were loaded onto a 12.5% SDS-polyacrylamide gel and sealed with agarose. Second-dimension electrophoresis was conducted using a Protean II XL multi cell (Bio-Rad, Hercules, CA, USA) at 16 mA gel⁻¹ for 30 min and then at 24 mA gel⁻¹ for 8 h or until the bromophenol blue had passed through the agarose/acrylamide interface. All of the gels were stained sequentially with Pro-Q Diamond fluorescent dye (Molecular Probes, Inc., Eugene, OR, USA) to detect phosphorylated proteins followed by Sypro Ruby (Molecular Probes, Eugene, OR, USA) to detect total proteins, as described by Chandramouli et al. (2011). The resulting protein patterns were captured using a Typhoon Trio imager (GE Healthcare, Piscataway, NJ, USA). All of the experiments included three independent replicates (n = 3). The scanned gels were analyzed using PDQuest Advanced software (version 8.0, Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Automatic spot detection in each gel was verified by manual inspection, and landmarks were assigned to align the different gels. The size of each spot was expressed as the spot volume. For each experimental condition, three 2-DE gels were matched to form a reference image. The reference gels from the control and treatment samples were matched so that the same number was assigned to the same spot in the different gels. The protein spots from the control and treated samples were considered significantly up- or down-regulated if they fulfilled the following criteria: p < 0.05 (Student’s t-test), a detection threshold average volume ≥ 20 (n = 3), and a differential fold change ≥ 2.0.

Identification of proteins by mass spectrometry

The gels used for Pro-Q Diamond and Sypro Ruby staining were then stained with Coomassie brilliant blue (Thermo Scientific, Rockford, IL, USA). Differentially expressed protein spots were excised, destained in 50 mM NH₄HCO₃ and 50% acetonitrile (ACN), digested with trypsin (12.5 ng μl⁻¹; Promega, Madison, WI, USA) and processed as described by Chandramouli et al. (2012). The digests were desalted with Zip-Tip (Millipore, Bellerica, MA, USA) and analyzed using an ABI4800 MALDI TOF/TOF™ analyzer (Applied Biosystems, Framingham, MA, USA). The mass spectra (MS) and tandem mass spectra (MS/MS) obtained were subjected to a MASCOT (Matrix Science, London, UK) search against the NCBI non-redundant sequence database (http://www.ncbi.nlm.nih.gov/RefSeq/). Taxonomy was restricted to bacteria to ensure that the protein entries identified had a bacterial origin. The database search was performed using the following criteria: mass tolerance of ±0.2 Da for peptides and ±0.5 Da for MS/MS ions; one missed cleavage and trypsin as the proteolytic enzyme; fixed modification = carbamidomethyl (cysteine), variable modification = oxidation (M), phosphorylation (ST); one tolerant missed cleavage; and a MOWSE score threshold of p < 0.05.

Metabolome analysis by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

Planktonic cells and biofilms were cultivated using the protocol described above for biofilm development. Bacterial cells were exposed either to 40 μg ml⁻¹ of poly-ether B for 30 min and 24 h (treated) or left unexposed (control). The cells were harvested together, centrifuged at 2700 × g and the resultant pellets were extracted 3 × with equal proportions of cold 50% methanol (MeOH) and dichloromethane (DCM). The cells were sonicated to break down the cell wall material and enhance the extraction efficiency. The two solvent layers were collected separately, evaporated until they were dry using a speed-vac (Thermo Electron, Waltham, MA, USA), dissolved in MeOH, and desalted using a Sep-Pak C₁₈ cartridge (Waters Corporation, Milford, MA, USA). The desalted samples were subjected to chemical characterization by UPLC-MS.

UPLC–MS acquisition and data analysis

The UPLC-MS analyses were performed using an Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled with a time-of-flight (TOF) mass spectrometer (Autoflex III, Bruker, Bremen, Germany) with an electrospray interface (ESI), as described by Dash et al. (2011). Crude extracts were dissolved in MeOH at a concentration of 10 μg ml⁻¹. Two ml of each sample were injected and metabolite separations were carried out using an Acquity UPLC column (BEH C₁₈: 2.1 mm × 150 mm, 1.7 μm; Waters Corporation, Milford, MA, USA) at 30 °C with the following solvent system: A = 0.1 vol. % formic acid–water, B = 0.1 vol. % formic acid–ACN. Gradient elution was performed at a flow rate of 0.25 ml min⁻¹ for 26 min, starting with 10% B moving to 100% B over 21 min and then holding at 100% B for 5 min. The MS in the m/z range of 150–1500 were analyzed using positive ionization mode with the ESI source at 200 °C and a detector voltage and cone voltage of 4500 V and 50 eV, respectively. All of the peaks exceeding a signal-to-noise ratio threshold of 5 with a maximum overlap of 3 compounds permitted were identified using an ESI Compass for the micro TOF workstation (Bruker, version 1.3). According to the retention time stability of an internal standard (sodium formate), the match window was set to 10 s. Peaks within the same retention time window were regarded as one peak and merged. Background noise was subtracted and the peak
signal was obtained from the raw data using the ‘find molecular features’ algorithm. The spectra from 30 and 24 min were bucketed, and the mass data were calibrated using sodium formate as an internal standard. Principle component analysis (PCA) and Profile Analysis 2.0 (Bruker, Bremen, Germany) were used to compare metabolite production in control and treated biofilms. The analysis involved comparisons both within (n=3) and between (n=2) batches.

Results

Dose-dependent effect of poly-ether B on the formation of biofilms

A two-way ANOVA revealed significant differences in biofilm formation between the control and poly-ether B treatments (F = 45.77, p < 0.0001; Figure 2). Exposure time accounted for 14.96% of the total variation (F = 83.96, p < 0.0001) while poly-ether B dosage accounted for 55.81% of the total variation (F = 313.2, p < 0.0001). The interaction between treatment time and dosage accounted for 24.47% of the total variation. After incubation with 20 and 200 μg ml⁻¹ of poly-ether B for 24 h the growth of the biofilm was reduced significantly to 38 and 46%, respectively. After incubation with 2 μg ml⁻¹ poly-ether B for 24 and 36 h, biofilms were reduced significantly (p < 0.0001) to 37 and 48%, respectively, cf. the control biofilms. At a concentration of 200 μg ml⁻¹, the formation of the biofilm was inhibited (35%) even after treatment for 30 min.

Reduction of biofilm thickness and growth inhibition by poly-ether B

Confocal laser scanning microscopy (CLSM) images revealed a marked reduction in the growth of biofilms of Vibrio sp. 010 in response to treatment with polyether B at 40 μg ml⁻¹ (Figure 3). Histograms plotted using Zen software and the respective z-stack measurements indicated that the control biofilms were thicker than those exposed to the 24 h treatment (Figure 3). COMSTAT analysis of CLSM images revealed that the thickness of the treated biofilm was substantially reduced (0.27 μm cf. the thickness of the control biofilm, 3.47 μm). The coverage of the substratum and the surface-to-volume ratio was also greatly reduced, 0.08 and 0.46 μm cf. control biofilm values of 1.00 and 1.25 μm, respectively (Table 1). To verify the results of the crystal violet binding assay and the confocal image analysis, bacterial cells were stained with differential viability dyes. Differential

Figure 2. The inhibitory effect of different concentrations of poly-ether B on the formation of biofilms by Vibrio sp. 010 over an exposure time of up to 36 h. The table shows the results of a two-way ANOVA with the treatment concentration and time as variables.

Figure 3. 2-D CSLM images of control and treated biofilms. The biofilms were grown in the presence of 40 μg ml⁻¹ poly-ether B for 24 h and stained with FITC-Con A. For each treatment n = 3. The image stacks of horizontal (x–y) optical sections were collected throughout the length of the biofilm and quantified using the COMSTAT program. The total intensity of the biofilm biomass was obtained by summing the intensity of all the x–y planes in the z-stack.
viability staining revealed that cells treated for 24 h had a significantly higher mortality \((F=1531, \ p<0.0001)\) (Figure 4b), whereas a 30 min treatment did not significantly \((p>0.05)\) reduce the number of viable bacterial cells (Figure 4a). The percentages of PI and FITC-stained bacterial cells in the presence of 40 \(\mu\)g ml\(^{-1}\) of poly-ether B after 30 min and 24 h are listed in Table S1. Treatment for 24 h resulted in 13.4% cell death, whereas in the controls, only 3.3% of the cells were dead \((p<0.0001;\) Figure 4b). Notably, cell mortality increased (13.4%) on increasing the exposure time to 24 h.

### Table 1. COMSTAT analysis of CLSM images.

|                   | Mean thickness (\(\mu\)m) | Roughness coefficient | Substratum coverage (%) | Surface to volume ratio (\(\mu\)m\(^2\); \(\mu\)m\(^3\)) |
|-------------------|---------------------------|-----------------------|-------------------------|----------------------------------|
| Control           | 3.47 ± 0.68               | 0.15 ± 0.05           | 1.00 ± 0.00             | 1.25 ± 0.08                       |
| Treated           | 0.27 ± 0.15               | 1.82 ± 0.04           | 0.08 ± 0.02             | 0.46 ± 0.04                       |

Note: Analysis based on images presented in Figure 3 of control biofilms and biofilms treated with 40 \(\mu\)g ml\(^{-1}\) of poly-ether B.

**Proteome and phosphoproteome response of the treated biofilm**

The proteomes of the control and treatment groups were separated using 2-DE. The resultant gels were stained with Sypro Ruby dye to detect total proteins and Pro-Q Diamond dye to detect phosphoproteins (Figure 5). The PDQuest software analysis revealed 772 and 651 protein spots in the control and treated biofilms, respectively. Following treatment, 59 spots showed significant changes \((p<0.05)\) in expression levels: 50 spots were up-regulated and 9 were down-regulated (Figure S2a). In

![Figure 4](image-url)  
**Figure 4.** Flow cytometric measurement of attached and planktonic cells stained with FITC and PI. Cells were treated with 40 \(\mu\)g ml\(^{-1}\) poly-ether B for 30 min (a) and 24 h (b). The cells were stained with PI and FITC. The dead and live bacterial populations (FITC and PI stained cells, respectively) were differentiated using a FACSria II flow cytometer equipped with a blue (488 nm) argon laser.
total, 126 and 168 phosphoprotein spots were detected in the control and treated biofilms, respectively. In response to treatment, 32 spots showed significant changes ($p < 0.05$) in expression levels: 11 spots were up-regulated and 21 were down-regulated (Figure S2b).

**The influence of poly-ether B on the expression of proteins involved in metabolism and the stress response**

Fourteen proteins, including four phosphoproteins, were identified and categorized as metabolism, membrane transport, glyoxylate pathway, and stress response proteins (Table 2). The observed MW and pI values of the identified proteins were very close to the theoretical values obtained from a MASCOT database search. Of the five metabolism proteins, phosphoribosylaminomimidazole synthetase (PurM; spot 3) was up-regulated in treated planktonic and biofilm populations, whereas ribose-phosphate pyrophosphokinase (RPPK; spot 7), branched-chain amino transferase (BCAT; spot 10), and putative endodeoxyribonuclease (PER; spot 11) were
down-regulated (Figure 6a). The transporter periplasmic substrate binding subunit (TRAP-T; spot 4) was highly up-regulated in treated cells. Enzymes involved in the anapleurotic glyoxalate pathway, such as malate synthase (MS; spot 1) and bifunctional 5,1-methylene tetrahydrofolate dehydrogenase (MTHF; spot 8) were down-regulated (Figure 6b). Poly-ether affected the expression of four stress response proteins, of which superoxide dismutase (SOD; spot 5) and demethyl ubiquinone-9, 3-methyl transferase (DMUT; spot 6) were up-regulated and chaperonin GroEL (GroEL; spot 2) was down-regulated (Figure 6c).

The influence of poly-ether B on the regulation of phosphoproteins

Six phosphoproteins (spots 12–17) were differentially expressed in response to treatment. Of these, two spots (spots 14 and 16) were expressed in insufficient abundance for the mass spectrometric analysis (Figure 7a). Four phosphoproteins were identified. Differentially expressed spots and the volume/intensity of each spot in the control and the treated gels are shown in Figure 7b. The ratio of phosphoprotein to the total protein intensity of each spot is shown in Figure 7c. Phosphoproteins such as ferrous trafficking protein (FTP; spot 15), Ti-type conjugative transfer relaxase Tra A (Tra A; spot 17), glyoxalase family protein (GP; spot 13), and deoxyribonucleic acid kinase (DNAk; spot 12), which participate in metabolism, membrane transport, the glyoxalate family, and stress responses, respectively, were down-regulated considerably with respect to total protein and phosphorylation levels. Unidentified phosphoprotein (spot 14) was up-regulated with respect to total protein and phosphoprotein levels in response to treatment, whereas an opposite trend was observed for spot 16 (Figure 7c).

The influence of poly-ether B on metabolites belonging to quorum-sensing and cell wall components

Scores and 2-D loading plots of the DCM (upper panel, a and b) and MeOH fraction (lower panel, c and d) for control and treated biofilms are shown in Figure 8. Differentially expressed metabolites in the DCM and MeOH fractions of treated biofilms are listed in Table 3. Samples that were treated with poly-ether B for 30 min clustered closely with those that were treated for 24 h and were slightly further away from control samples across the second principal component, which indicated that the bacterial metabolome responded quickly to treatment with poly-ether B. The PCA analysis and chromatogram of the DCM and MeOH fractions revealed that the production of several metabolites was affected.

| Spot no. | Accession no. | Protein identification | Theoretical MW/pI | Observed MW/pI | PM | SC (%) | Fold change |
|----------|---------------|------------------------|-------------------|----------------|----|--------|-------------|
| Metabolism proteins | | | | | | | |
| 3 | gi|219996731 | Phospho-ribosyl amino imidazole synthase | 37/4.6 | 39/4.3 | 3 | 100 | +2.4 |
| 7 | gi|71064752 | Ribose phosphate pyro phospho kinase | 34/5.7 | 36/5.7 | 12 | 99 | −5.5 |
| 10 | gi|71065773 | Branched chain amino transferase | 34/5.8 | 35/5.8 | 7 | 100 | −2.5 |
| 11 | gi|261492420 | Putative endodeoxy ribonuclease | 11/9.2 | 26/6.5 | 8 | 72 | −1.5 |
| 15 | gi|33301902 | Ferrous trafficking protein | 10/6.1 | 10/4.6 | 87 | 7 | NA* |
| Membrane transport proteins | | | | | | | |
| 4 | gi|71065307 | TRAP-T family transporter periplasmic substrate binding subunit | 36/4.6 | 36/4.6 | 5 | 99 | +8.3 |
| 17 | gi|154243865 | Ti-type conjugative transfer relaxase Tra A | 167/7.8 | 210/7.8 | 21 | 60 | −8.0* (−7.0) |
| Glyoxalate pathway proteins | | | | | | | |
| 1 | gi|296395112 | Malate synthase | 79/5.7 | 88/5.1 | 3 | 29 | −7.0 |
| 8 | gi|71065239 | Bifunctional 5,1-methylene tetrahydrofolate dehydrogenase | 31/5.6 | 33/5.9 | 6 | 100 | −2.1 |
| 13 | gi|260606492 | Glyoxalase family protien | 19/4.9 | 23/4.6 | 3 | 58 | +4.0* (−2.0) |
| Stress-associated proteins | | | | | | | |
| 2 | gi|93005371 | Chaperonin GroEL | 57/4.7 | 64/4.8 | 14 | 100 | −3.2 |
| 5 | gi|93007055 | Super oxide dismutase | 23/5.1 | 28/4.7 | 7 | 100 | +2.3 |
| 6 | gi|109895909 | De-methyl ubiquinone 9, 3 methyl transferase | 28/5.3 | 32/5.6 | 7 | 95 | +5.0 |
| 12 | gi|288943276 | DNAk | 69/4.6 | 70/4.4 | 2 | 33 | NA* |

Notes: The score had to be over the significance threshold (p < 0.05) for positive identification. Observed (Obs.) MW and pI values were estimated from 2-DE gels and Theoretical (Theo.) MW and pI values were derived from a database search by MASCOT. PM, number of peptides matching the protein sequence; SC, sequence coverage; *phosphorylation level; NA, not determined.
by the treatment. For example, in the DCM fraction, the tripeptides TrpPheGln \((R_t: 18.5 \text{ min})\) and GalNAc β1-4Glc NA cβ-sp \((R_t: 20 \text{ min})\) were produced at low levels while oleamide \((R_t: 21.9 \text{ min})\) was produced at high levels in treated biofilms (Table 3, DCM fraction; Figure S3). In the MeOH fraction, all three of the identified metabolites including 3-hydroxyl-butanoyl or homoserine lactone \((R_t: 4.1 \text{ min}; \text{HSL})\), AsnArg Tyr \((R_t: 17.5 \text{ min})\) and 1-hexadecylamine \((R_t: 15 \text{ min})\), were produced at low levels in treated biofilms (Table 3, MeOH fraction; Figure S4). However, these metabolites were produced abundantly in control biofilms. Hexadecylamine and HSL were identified as quorum-sensing molecules, whereas the tripeptides and the cell wall component GalNAc β1-4Glc NA cβ-sp were defined as ‘extracellular’ substances.

**Discussion**

Inhibition of biofilm development by natural products isolated from marine species has been well studied (Fusetani 2000; Kobayashi 2000; Qian et al. 2010). However, the molecular response mechanisms underlying this process have not been extensively studied. Cell viability assays and confocal analyses indicated that poly-ether B potentially induced a variety of physiological changes during the period from 12 to 24 h post-treatment. These changes included a reduction in the growth of the biofilm and the formation of a thin, weakly adherent biofilm. The attachment of bacteria to a substratum is crucial for biofilm development (Cunliffe et al. 1999). Antibiotics and biocides prevent biofilm formation by interfering with the attachment and adhesion of cells (Ashby et al. 1994; Allen et al. 2006). Results from the present study suggested that poly-ether B prevented surface conditioning that occurs at the beginning of the cell attachment stage. Differential staining was carried out to further assess the effect of poly-ether B on the viability of the bacterial cells. Flow cytometry is an effective tool for quantifying two populations of cells (control and treatment) that are discriminated by staining with fluorescent dyes such as PI and FITC (Kamau et al. 2000). A prolonged exposure (24 h) to poly-ether B increased cell mortality (13.4%), whereas a short exposure (30 min) resulted in only 0.2% mortality. The distinct increase in dead (PI-stained) cells following exposure for 24 h indicated that the cells were susceptible to poly-ether B. These findings were consistent with the cell viability data showing marked effects of exposure to poly-ether B at 40 μg ml⁻¹ for 24 h. Notably, control cells unexposed to poly-ether B for 24 h showed an increase in the intensity of the PI signal. Although PI has been used in the literature to assess cell viability (Nocker & Camper 2006), autofluorescence, and non-specific binding of PI should be considered when testing more complex samples such as biofilms.
as biofilms (Biggerstaff et al. 2006). The discrepancy indicating mortality of untreated cells might be a result of adherence of PI to the substratum resulting in an increase of the PI fluorescence signal. Although cell viability assays are useful and cost-effective methods, their ability to evaluate the molecular response of treated cells is limited. Since the effects of bioactive compounds are typically initiated via their interaction with biomolecules, it is essential to identify key proteins and metabolites that influence biofilm formation.

The proteome of biofilm and planktonic cells exposed to poly-ether B exhibited differential expression patterns. The proteins associated with nucleotide metabolism that responded to treatment with poly-ether B included PurM, RPPK, EDNase, BCAT, and FTP. PurM and RPPK are enzymes that are essential for purine anabolism and catabolism, respectively (Zientz et al. 2004; Rodionov et al. 2008). These enzymes may have enhanced the synthesis of nucleotide moieties that are required for the repair and replenishment of purines affected by the treatment. EDNase, BCAT, and FTP play an important role in the formation of biofilms. EDNase is required for the dispersal of target biofilms (Nijland et al. 2010), BCAT for the biosynthesis of branched-chain amino acids (Kaur et al. 2009) and FTP for the stimulation of biofilm formation (Wagner & Mulks 2006). The synthesis and differential expression of these proteins potentially facilitated biofilm colonization of smooth surfaces. Membrane proteins have a substantial influence on bacterial attachment to surfaces and on early biofilm development (Ritter et al. 2012). Poly-ether B affected the expression of two prokaryotic secondary transporters: TRAP-T (up-regulated) and Tra A (down-regulated). The physiological functions of these proteins in biofilm development remain unknown. Protein phosphorylation affects the attachment and colonization of bacterial pathogens by regulating the transcription of virulence genes that alter the exopolysaccharide content of the cell envelope (Charusanti et al. 2010). In V. cholera, phosphorylation of enolpyruvate phosphotransferase is essential for biofilm formation.
The down-regulation of phosphopryorylated proteins such as FTP, Tra A, DNAk, and GP suggests that poly-ether B also exerted a permissive effect on protein phosphorylation. Notably, two proteins, MS and MTHF, which participate in the glyoxylate cycle were down-regulated in response to treatment. In bacteria, the glyoxylate cycle is the dominant anaplerotic pathway for metabolome production (Wittmann et al. 2007), and is crucial for virulence and opportunistic colonization (Charusanti et al. 2010). Hence, it is reasonable to speculate that poly-ether B interfered with the glyoxylate cycle and thereby prevented bacterial cells from colonizing the substratum to form mature biofilms. The bacteria comprising a biofilm adapt to starvation and oxidative stresses by producing increased levels of intracellular oxidative stress proteins (Dukan & Nyström 1999). In V. cholera, GroEl regulates the synthesis of exopolysaccharide, thereby affecting biofilm architecture (Bomchil et al. 2003). Poly-ether B may interfere with this adaptation strategy by down-regulating the synthesis of stress
proteins. The present results showed that the synthesis of exopolysaccharide and stress adaptation regulates biofilm formation in a Vibrio sp.

MS-based metabolomics in conjunction with PCA analysis resulted in the identification of several metabolites that responded to poly-ether B treatment. The decreased production of quorum-sensing molecules, cell wall components, and extracellular tripeptides following treatment revealed specific cellular responses to the antifouling compound. Biofilm formation involves a coordinated series of molecular events that are controlled in part by quorum-sensing molecules (Davies et al. 1998). The quorum-sensing molecule HSL is known to induce the growth of biofilms in Vibrio spp. (Griffiths 2005; Yildiz & Visick 2009). Down-regulation of quorum-sensing molecules by poly-ether B likely inhibited the quorum-sensing system, which may result in a loosening of the biofilm architecture. Cell wall components may act as adhesins, permeability barriers and may also aid in the formation of biofilms (Kjelleberg & Givskov 2007). Cell wall moieties can act as signalling molecules that promote colonization by Shigella sp. (Nigro et al. 2008) and Bacillus sp. (Shah et al. 2008). Down-regulation of these component metabolites likely leads to a loss of membrane architecture, thereby affecting the attachment of cells. Hexadecylamine metabolites are cationic surface-active cariostatic agents that have beneficial effects on biofilm formation (van der Mei et al. 2008). The electrostatic interaction between cationic amines and the negatively charged bacterial cell surfaces is essential for surface adsorption and attachment. Oleamide, a lipid signaling molecule, may redirect the signaling mechanisms required for biofilm formation by elevating its level of production (Quist et al. 2000). However, the actual function of oleamide in this process remains to be elucidated.

In summary, the present work demonstrates the usefulness of an approach incorporating viability assays, proteomics, and GC–MS-based metabolomics to study the molecular responses of a Vibrio sp. biofilm to treatment with poly-ether B. Poly-ether B mainly caused changes in the expression of proteins that are involved in nucleotide metabolism, transport mechanisms, and the glyoxylate cycle. Metabolites affected by the treatment included quorum-sensing molecules, cell wall components, and surface-active agents. Down-regulation of proteins or metabolites by poly-ether B may lead to a loss of the ability of cells to attach and colonize a substratum thus preventing biofilm formation. Key molecular processes such as the glyoxylate cycle, the quorum-sensing system, nucleotide metabolism, and the stress response may serve as potential targets for antifouling compounds.

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Table 3. Differentially expressed metabolites identified in DCM and MeOH fractions of the poly-ether B treated biofilm.

| R<sub>t</sub> | m/z | Metabolite identification | Fold change |
|----------|-----|--------------------------|-------------|
| DCM fraction | | | |
| 18.5 | 479 | TrpPheGln | -2.3 |
| 18.3 | 453 | TrpPheGln (isomer) | -4.0 |
| 20 | 493 | Gal NAc β1-4Glc NA εβ-sp | -1.1 |
| 21.9 | 281 | Oleamide | +1.0 |
| MeOH fraction | | | |
| 4.1 | 187 | (3-Hydroxyl butanoyl) HSL | -1.1 |
| 17.5 | 451 | AsnArg Tyr | -1.1 |
| 15.0 | 241 | 1-Hyxadecylamine | -3.7 |

Notes: R<sub>t</sub>, retention time; m/z, mass-to-charge ratio.
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