The Fate of Marine Bacterial Exopolysaccharide in Natural Marine Microbial Communities

Zilian Zhang*, Yi Chen, Rui Wang, Ruanhong Cai, Yingnan Fu, Nianzhi Jiao*

State Key Laboratory of Marine Environmental Science, Institute of Marine Microbes and Ecospheres, Xiamen University, Xiamen, Fujian, People’s Republic of China

* jiao@xmu.edu.cn (NJ); zhangzilian@xmu.edu.cn (ZZ)

Abstract

Most marine bacteria produce exopolysaccharides (EPS), and bacterial EPS represent an important source of dissolved organic carbon in marine ecosystems. It was proposed that bacterial EPS rich in uronic acid is resistant to mineralization by microbes and thus has a long residence time in global oceans. To confirm this hypothesis, bacterial EPS rich in galacturonic acid was isolated from Alteromonas sp. JL2810. The EPS was used to amend natural seawater to investigate the bioavailability of this EPS by native populations, in the presence and absence of ammonium and phosphate amendment. The data indicated that the bacterial EPS could not be completely consumed during the cultivation period and that the bioavailability of EPS was not only determined by its intrinsic properties, but was also determined by other factors such as the availability of inorganic nutrients. During the experiment, the humic-like component of fluorescent dissolved organic matter (FDOM) was freshly produced. Bacterial community structure analysis indicated that the class Flavobacteria of the phylum Bacteroidetes was the major contributor for the utilization of EPS. This report is the first to indicate that Flavobacteria are a major contributor to bacterial EPS degradation. The fraction of EPS that could not be completely utilized and the FDOM (e.g., humic acid-like substances) produced de novo may be refractory and may contribute to the carbon storage in the oceans.

Introduction

The oceans are the largest carbon reservoir on the planet and hence have significant impacts on global climate. An important form of marine storage of carbon is as organic carbon, predominantly dissolved organic carbon (DOC) [1]. It is known that more than 95% of DOC is refractory dissolved organic carbon (RDOC), which is resistant to microbial utilization and can be stored for several thousand years in the oceans [2]. Marine microbes have been proposed as major contributors to the generation of RDOC; however, the processes and mechanisms involved in the formation of RDOC remain unclear [3].

Chemical analysis of DOC in the ocean indicates that polysaccharides are one of the major components of seawater DOC, accounting for up to 50% of the DOC in surface waters and up
to 25% of the DOC in deeper waters [4]. Among marine microbes, bacteria [5] and phytoplankton, such as diatoms [6], cyanobacteria [7] and dinoflagellates [8], are considered the major source of polysaccharides. Bacterioplankton represent the largest living surface in the world’s oceans and can exceed phytoplankton biomass even in the euphotic zone of oligotrophic regions [9]. It was indicated that capsular envelopes, which consist mainly of high-molecular-weight polysaccharides, are widely distributed in marine bacteria [5,10]. Now, numerous EPS producing bacteria have been isolated from marine environments, such as seawater, sediment, deep-sea hydrothermal vents, and sea ice [11]. EPS serves different functions in bacteria, such as the formation of a favorable microenvironment that facilitates attachment, maintenance of exoenzyme activity, sequestration of nutrients, and protection against toxins [12].

The composition of EPS varies considerably between phytoplankton and bacteria, which may in turn reflect its fate in the ocean. One of the major components of bacterial EPS is uronic acid, comprising up to 20–50% of the total polysaccharide fraction [13]. In contrast, phytoplankton EPS is relatively poor in uronic acid (< 5% of the total polysaccharide fraction) [13]. The negative charge associated with carboxyl groups of uronic acid of EPS has been implicated as a primary factor dictating complexation of these macromolecules with transition metals [13,14]. In turn, this property has been suggested to have a significant effect on the fate and ultimate degradation of EPS in the ocean [15]. As such, when compared to eukaryotic EPS, bacterial EPS is difficult to degrade and therefore tends to accumulate in marine environments [4,16]. However, factors influencing the bioavailability of bacterial EPS remain poorly understood.

The aim of this study was to clarify whether bacterial EPS rich in uronic acid is resistant to microbial utilization. To achieve this goal, we isolated EPS-producing bacteria from the South China Sea. The composition of EPS produced by JL2810, one of our marine bacterial isolates, was studied. EPS harvested from this organism was used to amend natural seawater, and the bioavailability of this EPS by native populations, in the presence and absence of ammonium and phosphate amendment, was investigated. Changes in the bacterial community structure during cultivation were also analyzed.

**Materials and Methods**

**Bacterial isolation and cultivation**

Seawater samples were collected from South China Sea during the cruise organized by National Natural Science Foundation of China on August 2012. Permit for the field study was obtained from National Natural Science Foundation of China. To isolate EPS-producing bacteria, aliquots (200 μL) of seawater were spread onto marine agar 2216E plates supplemented with 1.5% Agar. Morphologically different colonies were selected, inoculated into 4 mL of 2216E medium and incubated at 28°C for 3 days with agitation at 200 rpm, then supernatant was collected from the culture medium by centrifugation at 8,000 × g for 5 min. The content of the EPS in the supernatant was measured using the phenol/H2SO4 method with glucose (Glc) as the standard [17]. Among the isolates, bacterial strain JL2810, which was isolated from 75 m depth (115°E, 18°N), was found to produce large amount EPS and was selected as a representative strain for use in subsequence study.

The strain was maintained in a 2216E agar medium (MA; BD). A single colony of JL2810 was aseptically transferred to 2216E liquid medium and incubated at 28°C overnight with agitation at 180 rpm. After washing twice with 0.8% (w/v) NaCl, cells (20 mL) were inoculated into 2 L basal medium containing 1% Glc, 1.9% NaCl, 0.03% NH4Cl, 0.03% KCl, 0.04% K2HPO4, 0.05% MgSO4·7H2O, and 0.003% CaCl2·7H2O. The pH of the medium was 7.6. Cultivation was carried out at 25°C in a 3.7 L KLF 2000 bioreactor (Bioengineering, Wald,
Switzerland) which was aerated at 1–1.5 vvm with constant agitation at 500 rpm. The pH was controlled at 7.6 using 0.25 M NaOH and 0.25 M sulfuric acid.

**EPS production**

An aliquot (25 mL) of culture was collected each day from the bioreactor and 200 μL of this was used to measure the optical density (OD600) of the culture using a FlexStation 3 spectrophotometer (Molecular Device, USA). The remaining sample was filtered through a 0.22 μm Millex-GF filter unit and the supernatant was used to determine the concentration of Glc and EPS. Glc concentration was determined with a Lab Assay TM Glucose kit (Wako Pure Chemical Industries, Osaka, Japan). For quantification of EPS, three volumes of cold ethanol (-20°C) was added per volume of supernatant. The mixture was incubated overnight at 4°C and the precipitate was collected by centrifugation (12,000 × g, 30 min) and dissolved in deionized water.

**EPS isolation**

At the end of cultivation, 500 ml of sample was collected and centrifuged at 8000 × g for 20 min with an AllegraTM64R (Beckman-Coulter, Fullerton, CA, USA). The supernatant was collected and filtrated through a 0.45 μm filter and then a 0.22 μm filter. Three volumes of cold ethanol (-20°C) was added to one volume of supernatant and the mixture was incubated at 4°C overnight. The precipitate was harvested by centrifugation (12,000 × g, 30 min), washed with 75% ethanol three times, and then dissolved in water. After centrifugation (12,000 × g, 30 min), the EPS solution was dialyzed (3500 Da cutoff) against water for 24 h. After dialysis, EPS was re-precipitated with 3 volumes of ethanol at 4°C overnight and then harvested by centrifugation as described above. The crude EPS isolated was freeze dried and stored in a desiccator at room temperature. Crude EPS was dissolved in water in the concentration of 5 g/L and then was purified using anion-exchange chromatography with a DEAE-Sepharose Fast Flow column (1.6 × 30 cm) (GE Healthcare). EPS was eluted with 20 mL of H2O at a flow rate of 0.5 mL/min followed by a NaCl gradient (30 mL) that ranged from 0 to 2 M at a flow rate of 1 mL/min. The process was carried out on an AKTA purifier FPLC system (Amersham Biosciences). Each elution fraction (2 ml) was collected and the EPS content was assayed using the phenol/sulfuric acid method. Major EPS-containing fractions were combined. After dialysis against distilled water, the EPS was collected and lyophilized.

**Analysis of EPS composition**

Protein content of the EPS was quantified using the Coomassie brilliant blue G-250 protein assay [18]. Quantification of DNA was carried out using a PicoGreen DNA detection kit (QuantiFluorTM ds DNA, Promega). The C and N content was assayed with an elemental analyzer (PE 2400 II, Perkin Elmer).

The Molecular weight of the EPS was analyzed by high-performance liquid chromatography (HPLC). Separation was carried out on an Agilent 1260 Infinity HPLC system with refractive index detection via a TSK Gel G5000SWXL column (7.8 × 300 mm) and a TSK Gel G2000SWXL column (7.8 × 300 mm), connected in series. Mobile phase was 0.1 M ammonium acetate containing 0.02% sodium azide solution. The column temperature was set at 30°C and flow rate was 0.6 mL/min. Standard solutions including dextran (Mw: 511, 167, 67, 40, 10, 5 and 1KDa) and maltotetraose (Mw: 666.6), and purified EPS sample were prepared at the concentration of 10 mg/mL in mobile phase and injected in duplicate. The injection volume was 10 μL.
Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Sample (300 μg) was taken and added to separate tubes with 20 μg of inositol as internal standard. Methyl glycosides were prepared from the dry sample following the mild acid treatment by methanolysis in 1 M HCl in methanol at 80°C (16 hours), followed by re-N-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The sample was then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 hours). These procedures were carried out as described by Merkle and Poppe [19]. GC/MS analysis of the TMS methyl glycosides was performed on an Agilent 7890A GC interfaced to a 5975C MSD, using Agilent DB-1 fused silica capillary column (0.25 mm × 30m).

Analysis of EPS utilization
Surface seawater was collected from the Xiamen coast (118°14’E, 24°29’N) in March, 2014. To analyze the potential utilization of EPS, the crude EPS (0.08 g/L) was added alone (treatment label: EPS) or in combination with inorganic nutrients (NH₄Cl at a concentration of 26.7 mg/L and K₂HPO₄·H₂O at a concentration of 7.17 mg/L) (treatment label: EPS+N/P). For comparison, Glc (0.1 g/L) was also added alone (treatment label: Glc) or in combination with same amount of inorganic nutrients (treatment label: Glc + N/P). The C:N:P ratio was initially 106:16:1. Supplement of EPS (0.08 g/L), Glc (0.1 g/L) and same amount of inorganic nutrients (EPS+Glc+N/P) was also performed. Seawater without any amendment served as control. Each treatment was performed in two replicates. Two hundred mL of each treatment was performed in 500 mL Erlenmeyer flasks and these were incubated at 25°C with agitation at 160 rpm for 14 days. During cultivation, samples (4 ml) were taken at 12 h intervals for the first 5 days and at 24 h intervals during the rest of the cultivation period. The microbial growth (OD₆₀₀), total sugar content, and Glc content of the samples were analyzed. OD₆₀₀ and Glc content were measured as described above. Total sugar in the dissolved sample was measured using the phenol/H₂SO₄ method with Glc as the standard [17]. At the end of cultivation, samples (30 mL) were filtered (0.22 um, Milliex-GP Filter Unit) into Silicon boride bottles (pre-combusted, 450°C, 4 h) and stored at -20°C for fluorescent dissolved organic matter (FDOM) determination.

Fluorescence measurement
Fluorescence measurement for FDOM was performed with fluorescence spectrophotometer (Cary Eclipse, Varian, USA) under a high sensitivity mode of 10 nm slit widths (excitation and emission) [20]. The Excitation Emission Matrix (EEM) measurement and normalization of fluorescence intensity were conducted according to methods described by Stedmon [21]. EEMs were generated by scanning emission spectra from 250 nm to 560 nm at 2 nm intervals, with 5 nm increments of the excitation wavelength from 200 nm to 450 nm. The EEM of Mill-Q water was subtracted from each sample and then EEMs were normalized to Raman units. The analysis was carried out in MATLAB with a “N-way toolbox for MATLAB” and split-half analysis which was used to validate the identified components.

DNA extraction and PCR amplification
Microbial genomic DNA was extracted from 2 and 14 days’ cultures of each treatment using a PowerMax Soil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. The resulting DNA (~ 2 μg) from each sample was purified and concentrated via ethanol precipitation. DNA concentration was estimated using a
NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and analyzed through gel electrophoresis. Q5 High-Fidelity DNA Polymerase (NEB) was used for the amplification of the V4 hypervariable region of 16S rRNA gene from microbial genome DNA using the universal primers (forward primer: 5′-AYTGGGYDTAAAGNG—3′ and reverse prime: 5′-TACNVGGGTATCTAATCC- 3′). The PCR reaction conditions were as follows: initial denaturation at 98°C for 5 min, followed by 27 cycles of 98°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and extension at 72°C for 5 min. PCR product was excised from a 1.5% agarose gel and purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union, CA, USA).

Illumina sequencing and 16S rDNA-based taxonomic analysis

The V4 amplicon was sequenced using pair-end method by Illumina Miseq with a 6 cycle index read. The average length of sequence reads was 221 bp. Sequence reads were assembled by Flash software (http://www.genomics.jhu.edu/software/FLASH/index.shtml) and the reads which could not be assembled were discarded. Only reads that had quality value (QV) scores of 20 for more than 99% of the sequence were extracted for further analysis. Chimera sequences were identified and removed using UCHIME. Sequences clustering was performed by UCLUST (QIIME) with a similarity cutoff of 97%, and clustered into operational taxonomic units (OTUs). The longest sequence in each cluster was chosen to be the representative sequences, which were annotated by RDP-classifier 2.2 (QIIME). The nucleotide sequences were submitted to GenBank under accession number SRP047314.

Results

Production of EPS by Alteromonas sp. JL2810

EPS-producing bacteria were isolated from seawater collected from the South China Sea. Among the isolates, strain JL2810, which produced the highest level of EPS, was selected for further use in this study. The 16S rRNA gene sequence of JL2810 exhibited 99.6% and 99.4% homology to that of Alteromonas macleodii ATCC27216T (CP003841) [22] and Alteromonas marina SW-47(T) (AF529060) [23], respectively. Therefore, JL2810 was identified as a strain of Alteromonas, and was designated Alteromonas sp. JL2810.

To analyze the EPS production, JL2810 was cultivated in a bioreactor at constant pH, dissolved oxygen and temperature. Glc was used as the sole carbon source. As shown in Fig 1, Glc was rapidly used by JL2810 cells, and complete utilization was observed by the fifth day. EPS was produced by JL2810 during the growth period and not reused by the cells at the late growth phase. At the end of cultivation, about 8% of Glc was converted to EPS, which reached a maximum of 0.77 g/L in the medium. Analysis of the EPS produced by JL2810 indicated it was primarily composed of carbohydrates, which accounted for 65% (w/w) of the EPS. Only a small amount of protein (0.92%) and DNA (1.08%) were detected. Elemental analysis revealed that C accounted for 33.84% of the total EPS mass, while N accounted for 0.88%. The C/N ratio of the EPS is about 38.

The molecular weight of the EPS is higher than 1.67 × 10^5 Da, which is similar to the molecular weight of EPS (range 1–3 × 10^5 Da) produced by most marine bacteria [11]. Analysis of the monosaccharide composition of the EPS indicated that the EPS contained a large amount of galacturonic acid (42.2%) and neutral monosaccharide including mannose (24.6%), rhamnose (16.2%), and Glc (14.4%). The high concentration of galacturonic acid in EPS produced by JL2810 is similar to that produced by other strains of Alteromonas, which were previously shown to contain 37–42% uronic acid [24].
Utilization of EPS by native marine communities

To analyze the bioavailability of bacterial EPS, bioassay experiments were conducted. The EPS and Glc were added to natural seawater, either alone or in combination with inorganic nutrients of N and P, and the effect of these amendments on microbial growth was determined. As shown in the Fig 2A, no microbial growth was observed in unamend controls. In contrast, microbial growth (OD600) in seawater amended with Glc or Glc + N/P was about two times higher than that amended with EPS or EPS + N/P. Maximum growth was achieved in 1–2 d in seawater that was amended with Glc, whereas growth peaks were detected after 3 and 6 days of cultivation in seawater amended with EPS (Fig 2A). This result indicates that, when compared to Glc, the EPS was less bioavailable or was not as readily used for microbial growth.

Inorganic nutrient (N/P) amendment stimulated microbial growth, especially with Glc as the carbon source. A slight increase in microbial growth was also observed in EPS amended seawater with added N and P (EPS + N/P). As shown in the Fig 2A, microbial growth was nearly the same during the first four days of cultivation with EPS or EPS + N/P and was slightly increased by the addition of the inorganic nutrients N and P during later growth stages. Among the treatments examined, cell density in the EPS + Glc + N/P treatment was the highest.

Consumption of Glc and EPS

Glc and total sugar concentrations were monitored during the cultivation period. As shown in Fig 2B, Glc was completely consumed within one day in the Glc + N/P treatment. When Glc
alone was added, the consumption was slower, and ~20% of Glc remained at the end of the growth period. Total sugar concentrations in the two EPS treatments (EPS and EPS + N/P) decreased in a similar manner over the first five days of cultivation and were higher in the EPS treatment than in the EPS + N/P treatment from five to fourteen days (Fig 2B). At the end of cultivation, 27% of the added sugar remained in the EPS treatment, while only 8% remained in
the EPS + N/P treatment. This result indicates that the consumption of both EPS and Glc are promoted by the addition of N and P.

Production of FDOM

Spectroscopic techniques provide a relatively simple method for tracking changes in the bulk chemical composition of DOM. In particular, the optically active fraction of DOM that fluoresces is inherently related to the chemical and structural composition of DOM [25]. The interpretation of EEM derived from fluorescence spectroscopy has been developed substantially with the aid of a powerful multivariate statistical approach called parallel factor (PARAFAC) analysis [26]. According to PARAFAC modeling analysis, three FDOM components can be detected in the culture medium at the end of the incubation: two humic-like components (components 1 and 2) and one protein-like component (component 3) (Fig 3A). The chemical properties of component 1 (excitation/emission wavelength (Ex/Em) of 312 nm/380-420 nm) are similar to those of terrestrial humic-like components. Likewise, component 2 (Ex/Em of 340 nm/440 nm) was similar to marine humic-like components. The fluorescence of component 3 (Ex/Em of 280 nm/350-420 nm) was similar to a protein-like component [27]. Compared with the control treatment, the fluorescence intensities of components 1 and 2 in Glc and Glc + N/P groups were greater (Fig 3B). Fluorescence intensities of the two humic-like

Fig 3. Three fluorescence components identified by the PARAFAC model. (A) Fluorescence graphs of the three components (component 1–3). (B) Fluorescence intensities of the three components after cultivation of seawater for 14 days.

doi:10.1371/journal.pone.0142690.g003
components in the EPS group were relative high and were reduced when seawater was amended with N/P. The fluorescence intensities of components 1 and 2 were the highest in the EPS + Glc + N/P treatment, and the values were nearly twice as intense as the individual EPS and Glc treatments. Except for the EPS treatment, the fluorescence intensity of component 3, a protein-like component, was low in all treatments when compared to the control. These results indicated that components 1 and 2 (humic acid-like) are highly produced during cultivation when compared to component 3 (protein-like).

Changes in bacterial community structure
To understand the microbial community involved in the utilization of EPS and Glc, the bacterial community structures in the 2-day (d) and 14-d incubations of each treatment were analyzed. The rarefaction curves obtained for the V4 region of 16S rRNA gene sequences nearly reached saturation for many samples (S1 Fig). After the quality control, a total of 588,448 validated sequence reads were obtained, which were classified into a total of 6,546 different OTUs based on an identity level of 97%. The coverage ranged from 97.8–98.7%, suggesting that the number of sequences in each samples was enough to reflect the real bacterial diversity. Of these OTUs, 5486 and 4606 OTUs were classified as phylum and class levels, respectively.

We classified the OTUs based on their phylogenetic affiliations and determined their relative proportions in the total reads. In 2-d cultures, the phylogenetic affiliation of microbes in the control was assigned to 21 bacterial and one archaeal phyla, with the dominant phyla being Proteobacteria (55.1%), Bacteroidetes (Cytophaga-Flavobacteria-Bacteriodes) (26.0%), Actinobacteria (6.1%), Verrucomicrobia (1.8%) and Planctomycetes (1.5%) (S2 Fig). The minor phyla (<1% of total microbial sequences) (Other, S2 Fig) were Euryarchaeota, Acidobacteria, Aquificae, Bacteria_incertae_sedis, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospira, OD1, Spirochaetes, Tenericutes, and WS3. The bacterial community composition changed dramatically from Proteobacteria toward Bacteroidetes in the 2-d cultures supplemented with EPS (S2 Fig). Bacteroidetes accounts for 67% (EPS) and 56% (EPS + N/P) of the total microbial sequences (S2 Fig). Flavobacteria was identified as the major contributor of the Bacteroidetes response to EPS treatment, followed by Sphingobacteria. The Flavobacteria accounts for 41% and 38% of total microbial sequences in EPS and EPS + N/P, while Sphingobacteria accounts for 21% and 13% (Fig 4A), respectively. In the three Glc supplemented cultures, on the other hand, the Proteobacteria phylum was the predominant microbial group, with members of the four major classes of Alpha-, Beta-, Gamma-, and Epsilonproteobacteria accounting for 87–92% of the total microbial sequences (Fig 4A). Gammaproteobacteria, accounting for 70–76% of the total microbial sequences, was identified as the predominant class of Proteobacteria in the Glc, Glc + N/P, and EPS + Glc + N/P cultures (Fig 4A). Compared to the EPS and Glc alone treatments, the Alphaproteobacteria and Gammaproteobacteria classes were relatively abundant when inorganic nutrients (N and P) were supplemented. Other groups were rare, and many sequences were present at abundance < 1% of the total population (Other, Fig 4).

In the 14-d cultures, Bacteroidetes (53%) was the predominant microbial group only in the culture sample supplemented with EPS, while Proteobacteria (35%-74%) was the predominant microbial group in other samples (Fig 4A). The relative abundance of Flavobacteria dramatically decreased in the 14-d cultures (4.5%, on average) amended with EPS (EPS and EPS + N/P) than that in 2-d cultures (40%, on average). Gammaproteobacteria also decreased in the seawater supplemented with Glc (Glc, Glc + N/P and EPS + Glc + N/P) from about 70% (in 2-d cultures, on average) to 1% (in 14-d cultures, on average). At the end of cultivation, the relative abundance of Sphingobacteria of Bacteroidetes was relatively higher than in the control. The data indicated
Fig 4. Bacterial community composition analysis. Phylogenetic compositions of the bacteria in the 2-d (2D) and 14-d (14D) cultures were analyzed. The relative abundance of each class is expressed as the mean percentage of total sequences obtained in the treatment. The major classes (A) and genus belonging to Flavobacteria class (B) and Gammaproteobacteria class (C) were indicated.

doi:10.1371/journal.pone.0142690.g004
that the dynamic change in the bacterial community structure responded to the carbon source present during cultivation; also, the members of Flavobacteria are important for the utilization of EPS, while those of Gammaproteobacteria are important for the utilization of Glc.

Due to the overwhelming sequence abundance, the composition of the classes of Flavobacteria and Gammaproteobacteria were scrutinized in more detail. Two families of Flavobacteriaceae and Cryomorphaceae, belonging to Flavobacteria, were identified in the cultures, with Flavobacteriaceae as the predominant group (Fig 4B). The genus Arenibacter in the family Flavobacteriaceae specifically responded to EPS treatment. The unclarified Flavobacteriaceae accounts for 15% (EPS) and 25% (EPS + N/P) of the sequences in the 2-d cultures (Fig 4B). Two genera Pseudoalteromonas and Vibrio, belonging to Gammaproteobacteria, were predominant in the Glc supplemented cultures (Fig 4C), indicating that growth of the members of these genera were specifically stimulated by Glc.

**Discussion**

Heterotrophic microbial communities are responsible for re-mineralizing and transforming a considerable fraction of autochthonous marine DOC [28] that comprise most of the organic matter in marine systems [29]. These communities are active components in the marine pelagic food web, leading to an efficient cycling of carbon and energy in the microbial loop [30]. They convert labile DOC into biomass, CO₂, and RDOC that can be stored for long periods in global oceans. Members of the genus Alteromonas are increasingly recognized as an active component of marine communities. They are globally distributed in the ocean and have a copiotrophic way of life, and as γ-strategists, they can grow rapidly when fresh carbon is released into marine habitats [31,32]. Strains of Alteromonas typically produce EPS [33,34] and are free living or associated with particles [31,35]. In the South China Sea, Alteromonas was isolated from seawater samples from all depths and identified as a dominant taxon (data not shown).

The EPS producing strain JL2810 was selected for this study. The strain converted about 8% Glc to EPS within five days of cultivation. The EPS produced by JL2810 had a molecular weight > 1.67 × 10⁵ Da and contained large amount (42.2%) of galacturonic acid as uronic acid sugar, which is similar to that produced by most marine bacteria. Therefore, the EPS used in this study is likely a representative of bacterially produced EPS in marine systems.

Our data indicated that EPS is less efficient for microbial growth than the monosaccharide Glc, suggesting that the bioavailability of carbohydrates depends partly on their chemical composition. Previous studies suggested that only small molecules, less than ~600 Da [36], can be transported across the cellular membrane. There is evidence that the incorporation rate of large molecules of bacterial capsular material from bacterioplankton is lower than the corresponding rates for Glc uptake [37]. Therefore, the high molecular weight EPS must be hydrolyzed to small molecular organic matter prior to its transport and use inside the cell; hydrolysis is a recognized rate-limiting step in the microbial utilization of DOM [38]. The enzymes for hydrolysis of EPS are usually highly specific, and their specificity is determined by carbohydrate structure, with uronic acids often playing a major role [39]. The EPS produced by JL2810 is a heteropolysaccharide containing mannose, rhamnose, Glc, galactose, and a relatively large amount of galacturonic acid. Thus, the hydrolysis of this EPS might require a diverse set of enzymes [39].

The EPS could not be completely utilized during the 14 d cultivation experiments, suggesting that the remaining EPS molecules might be difficult for microbes to utilize. To confirm this hypothesis, a longer incubation would need to be used. The composition and molecular features of the remaining EPS molecules also need to be analyzed. Consumption of Glc was largely promoted by inorganic nutrients (Glc+N/P). On the other hand, the consumption of EPS only
was promoted at the late growth stages (Fig 2B), suggesting that EPS may contain N and P itself; so, the N and P are not the limiting factors during the initial utilization of EPS but are likely to become limiting during later stages of breakdown. Similarly, it was indicated that N and P contained in algal organic material was important for bacterial growth and the utilization of the organic material [40]. In support of this hypothesis, a small amount of protein and DNA was detected in the EPS, which may provide N and P for microbial growth. It may also suggest that there is a lag time before a natural population can utilize EPS or the microbes that can use EPS take longer to grow and become more abundant later in the experiment. Without nutrient supplementation, both Glc and EPS cannot be completely utilized by the microbial assemblages. These results indicate that the bioavailability of DOC is not only determined by its composition but is also affected by inorganic nutrient availability. When inorganic nutrients are replete, DOC can be mobilized for degradation [41]. The increased microbial growth in the presence of inorganic N and P might account for the increased Glc and EPS consumption. EPS production usually increases when marine bacteria are grown in laboratory conditions with a limited availability of nutrients, such as nitrogen and phosphorus [42]. Taken together, these data suggest that inorganic nutrient limitation may shift DOC towards longer residence times in the ocean.

Consumption of Glc and EPS was linked to the production of humic-like components 1 and 2 (Fig 3). As shown in Fig 4, Glc and EPS were utilized by different bacterial community, suggesting that different humic acid-like components might be produced in Glc and EPS treatment incubations. Component 1 is often used as a tracer for terrestrial CDOM in coastal environments, but it can originate from either terrestrial or marine microbial sources [43]. Component 2 has been widely observed in the seawaters and is hypothesized to originate from autochthonous processes [44]. Production of component 2 by bacteria was also indicated by Romera-Castillo [45]. Humic-like components have been traditionally considered refractory components that resist bacterial degradation and can be accumulated in the ocean on centennial to millennial time scales [46,47]. The protein-like component 3 decreased in fluorescence intensity during cultivation in all treatments, with the exception of EPS treatment. Relatively higher intensities of component 3 detected in the EPS group may partly originate from the EPS itself; the addition of N and P promoted EPS degradation and reduced the fluorescence of this component (Fig 3). This hypothesis was supported by the identification of protein-like fluorescence substance with Ex/Em similar to the component 3 in extracellular polymeric substances [48]. Analysis of the distribution of FDOM in the ocean revealed that the fluorescence intensity levels of the protein-like component were highest in the surface waters and decreased with depth, but did not change systematically in the bathypelagic layer (1000 m-bottom). However humic-like components were lowest in the surface and increased with depth in the mesopelagic layer (200–1000 m), and then slightly decreased with depth in the bathypelagic layer [20,43]. These result suggest that component 3 is more bioavailable and is more readily used than humic-like components [43]. Thus, the formation of humic-like components during the consumption of bacterial EPS might contribute to the RDOC pool in the ocean.

Microbial community structure analysis indicated that Proteobacteria and Bacteroidetes are the major phyla involved in the consumption of monosaccharide Glc and bacterial EPS, respectively (S2 Fig). It is known that Proteobacteria and Bacteroidetes are the most abundant heterotrophic bacteria in the ocean [49]. The bacterial community composition changed dramatically from Proteobacteria towards Bacteroidetes after 2 d cultivation with EPS as carbon source (S2 Fig). In fact, representatives of Bacteroidetes have been increasingly recognized as specialists for the degradation of macromolecules. For example, it has been indicated that the Flavobacteria and Sphingobacteria of Bacteroidetes often appear in oil-polluted seawater, indicating their ability to degrade relatively refractory hydrocarbons [50]. The relative abundance of
Flavobacteria was about 40% in the community structure of the 2-d culture supplemented with EPS, but decreased to about 5% in the 14-d culture. The result suggested that the Flavobacteria specifically responded to the EPS treatment and that they may be involved in the hydrolysis of EPS. It is known that the members of Flavobacteria are especially proficient in degrading various biopolymers, such as cellulose, chitin, and pectin [51] and thus make a significant contribution to the remineralization of marine organic matter [49]. In addition, Flavobacteria isolated from lake water decompose refractory substrates more efficiently than do other strains such as Pseudomonas and Erwinia belonging to the phylum Proteobacteria [52]. Genomic analysis indicated that the marine flavobacterium Formosa agariphila KMM 3901T has the capacity to degrade a wide range of algal polysaccharides [53]. Further growth experiments confirmed that the strain can use abundant algal polysaccharides [53]. Enzymes involved in the degradation of polysaccharides from the cell walls of green algae rich in uronic acid were also identified from marine bacteria of Flavobacteria [54]. Genomic analysis indicated that marine flavobacterium Formosa agariphila KMM 3901T has the capacity to degrade a wide range of algal polysaccharides [53]. Further growth experiments confirmed that the strain can use abundant algal polysaccharides [53]. Enzymes involved in the degradation of polysaccharides from the cell walls of green algae rich in uronic acid were also identified from marine bacteria of Flavobacteria [54]. In our study, the genus Arenibacter, belonging to the class Flavobacteria, was abundant in the cultures supplemented with EPS (Fig 4B). Recently, it was indicated that the strains of the genus Arenibacter can synthesize a wide spectrum of glycosidases, especially β-N-acetylglucosaminidases and α-N-acetylgalactosaminidases, and may participate in the degradation of natural polysaccharides in marine environments [55]. The EPS used in this study was isolated from marine bacterium JL2810 and is rich in galacturonic acid; therefore, the enzymes synthesized by members of Arenibacter might be important for the utilization of the EPS. The growth of genus Bizonia was specifically promoted in the 2-d culture amended with EPS (Fig 4B). The genus Bizonia was first described by Nedashkovskaya et al. [56], and since then, few species have been isolated. The utilization of EPS by these species has not been described. The role of the members of Bizonia in the ocean carbon cycle is largely unknown. The mechanism of growth of Bizonia in the culture supplemented with EPS without N/P need to be further addressed. In this study, we only analyzed the bacterial community composition in 2- and 14-d cultures, since maximum bacterial growth was detected in 2-d culture in preliminary experiment amended seawater with Glc. However, we noticed that growth peaks were also detected after 3 and 6 days of cultivation in seawater amended with EPS, suggesting that bacterial community composition may changed significantly during the cultivation in EPS treatment groups.

When Glc served as the carbon source, the Gammaproteobacteria class was the dominant bacteria, suggesting that the members of Gammaproteobacteria are more sensitive to the labile DOC such as Glc. Two genera Pseudoalteromonas and Vibrio belonging to the class Gammaproteobacteria were predominant in the Glc-supplemented cultures (Fig 4C). This result is consistent with the previous observations that the copiotrophic organisms, often belonging to clades within the Gammaproteobacteria (e.g. Vibrio, Alteromonas, Pseudoalteromonas), could rapidly reach numerical dominance within labile DOM (e.g. Glc, gluconic acid) supplemented treatments [57] and are considered opportunistic r-strategists because of their short generation times and rapid growth response [58].

Conclusions

This study showed that bacterial EPS rich in uronic acid is less efficient for microbial utilization than the monosaccharide Glc. Consumption of EPS and Glc was promoted by the inorganic nutrients N and P. Flavobacteria and Gammaproteobacteria are the major contributors to the utilization of EPS and Glc, respectively. The humic-like components synthesized de novo and the fraction of EPS that cannot be hydrolyzed might contribute to the formation of RDOC in the ocean. The structure and composition of the EPS that remained in the culture system need to be further analyzed in the future.
Supporting Information

S1 Fig. Diversity of microbial communities. The total numbers of screened clones are plotted against the unique operational taxonomic units (OTUs) in the 2-d (2D) and 14-d (14D) culture samples. (PPTX)

S2 Fig. Bacterial community composition analysis at phylum level. (PPTX)

Acknowledgments
We thank the cruise supported by the National Research Foundation of South China Sea on August 2012.

Author Contributions
Conceived and designed the experiments: ZZ NJ. Performed the experiments: YC RW RC. Analyzed the data: ZZ YC RW RC YF. Contributed reagents/materials/analysis tools: ZZ YC RW RC YF. Wrote the paper: ZZ YC RW.

References
1. Reeburgh WS. Figures summarizing the global cycles of biogeochemically important elements. Bull Ecol Soc Am. 1997; 78: 260–267.
2. Eichinger M, Poggiale JC, Van Wambeke F, Lefevre D, Sempere R. Modelling DOC assimilation and bacterial growth efficiency in biodegradation experiments: A case study in the Northeast Atlantic Ocean. Aquatic Microbial Ecology. 2006; 43: 139–151.
3. Jiao N, Herndl GJ, Hansell DA, Benner R, Kattner G, Wilhelm SW, et al. Microbial production of recalcitrant dissolved organic matter: long-term carbon storage in the global ocean. Nature Rev Microbiol. 2010; 8: 593–599.
4. Benner R, Pakulski JD, McCarthy M, Hedges JI, Hatcher PG. Bulk chemical characteristics of dissolved organic matter in the ocean. Science. 1992; 255: 1561–1564. PMID:17820170
5. Heissenberger A, Leppard GG, Herndl GJ. Relationship between the intracellular integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. Appl Environ Microbiol. 1996; 62: 4521–4528. PMID:16535466
6. Myklestad S. Production of carbohydrates by marine planktonic diatoms. II. Influence of the NP ratio in the growth medium on the assimilation ratio, growth rate, and production of cellular and extracellular carbohydrates by Chaetoceros affinis var. willei (Gran) Hustedt and Skeletonema costatum (Grev.) Cleve. J Exp Mar Biol Ecol. 1977; 29: 161–179.
7. Singh N, Asthana R, Kayastha A, Pandey S, Chaudhary A, Singh S. Thiol and exopolysaccharide production in a cyanobacterium under heavy metal stress. Process Biochem. 1999; 35: 63–68.
8. Brachvogel T, Schweitzer B, Simon M. Dynamics and bacterial colonization of microaggregates in a large mesotrophic lake. Aquatic Microbial Ecology. 2001; 26: 23–35.
9. Cho BC, Azam F. Biogeochemical significance of bacterial biomass in the oceans euphotic zone. Mar Ecol Prog Ser. 1990; 63: 253–259.
10. Sutherland IW. Bacterial exopolysaccharides. Adv Microb Physiol. 1972; 8: 143–213. PMID:4581483
11. Poli A, Anzelmo G, Nicolaus B. Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. Marine Drugs. 2010; 8: 1779–1802. doi:10.3390/md8061779 PMID:20631870
12. Decho AW. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanogr Mar Biol Annu Rev. 1990; 28: 73–153.
13. Bhaskar P, Bhosle NB. Microbial extracellular polymeric substances in marine biogeochemical processes. Current Science. 2005; 88: 45–53.
14. Kennedy AF, Sutherland IW. Analysis of bacterial exopolysaccharides. Biotechnol Appl Biochem. 1987; 9: 12–19. PMID:3032214
Tranvik LJ. Microbial transformation of labile dissolved organic matter into humic-like matter in seawater. FEMS Microbiol Ecol. 1993; 12: 177–183.

Gutierrez T, Berry D, Yang T, Mishamandani S, McKay L, Teske A, et al. Role of bacterial exopolysaccharides (EPS) in the fate of the oil released during the deepwater horizon oil spill. PLoS One. 2013; 8: e67717. PMID: 23826336

Tranvik LJ. Microbial transformation of labile dissolved organic matter into humic-like matter in seawater. FEMS Microbiol Ecol. 1993; 12: 177–183.

Dubois M, Gilles KA, Hamilton JK, Rebers P, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956; 28: 350–356.

Sedmak JJ, Grossberg SE. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Analty Biochem. 1977; 79: 544–552. PMID: 68686

Merkle RK, Poppe I. Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/mass spectrometry. Methods Enzymol. 1994; 230: 1–15. PMID: 8139491

Yamashita Y, Tsukasaki A, Nishida T, Tanoue E. Vertical and horizontal distribution of fluorescent dissolved organic matter in the Southern Ocean. Mar Chem. 2007; 106: 498–509.

Stedmon CA, Bro R. Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. Limnol Oceanogr: Methods. 2008; 6: 1–8.

Baumann L, Baumann P, Mandel M, Allen RD. Taxonomy of aerobic marine eubacteria. J Bacteriol. 1972; 110: 402–429. PMID: 4552999

Yoon JH, Kim IG, Kang KH, Oh TK, Park YH. Alteromonas marina sp. nov., isolated from sea water of the East Sea in Korea. Int J Syst Evol Microbiol. 2003; 53: 1625–1630. PMID: 13130060

Ragueneau G, Cambon-Bonavita MA, Lohier JF, Boisset C, Guenezennec J. A novel, highly viscous polysaccharide excreted by an Alteromonas isolated from a deep-sea hydrothermal vent shrimp. Curr Microbiol. 2003; 46: 448–452. PMID: 12732953

Kothawala DN, von Wachenfeldt E, Koehter B, Tranvik LJ. Selective loss and preservation of lake water dissolved organic matter fluorescence during long-term dark incubations. Sci Total Environ. 2012; 433: 238–246. doi: 10.1016/j.scitotenv.2012.06.029 PMID: 22796414

Andersen CM, Bro R. Practical aspects of PARAFAC modeling of fluorescence excitation emission data. J Chemomot. 2003; 17: 200–215.

Coble PG, Del Castillo CE, Avril B. Distribution and optical properties of CDOM in the Arabian Sea during the 1995 Southwest Monsoon. Deep-Sea Research Part II. 1998; 45: 2195–2223.

Azam F. Microbial control of oceanic carbon flux: The plot thickens. Science. 1998; 280: 694–696.

Hedges JI. Global biogeochemical cycles: progress and problems. Mar Chem. 1992; 39: 67–93.

Ivars-Martinez E, Martin-Cuadrado AB, D’Auria G, Mira A, Ferreira S, Johnson J, et al. Comparative genomics of two ecotypes of the marine planktonic copiotroph Alteromonas macleodi suggests alternative lifestyles associated with different kinds of particulate organic matter. ISME J. 2008; 2: 1194–1212. doi: 10.1038/ismej.2008.74 PMID: 18670397

McCarren J, Becker JW, Repeta DJ, Shi Y, Young CR, Malmstrom RR, et al. Microbial community transcriptomes reveal microbes and metabolic pathways associated with dissolved organic matter turnover in the sea. Proc Natl Acad Sci USA. 2010; 107: 16420–16427. doi: 10.1073/pnas.1010732107 PMID: 20807744

Rougeaux H, Talaga P, Carlson RW, Guenezennec J. Structural studies of an exopolysaccharide produced by Alteromonas macleodi subsp. fijensis originating from a deep-sea hydrothermal vent. Carbohydr Res. 1998; 312: 53–59. PMID: 9836450

Roger O, Kervarec N, Ratsiskol J, Colliec-Jouault S, Chevolot L. Structural studies of the main exopolysaccharide produced by the deep-sea bacterium Alteromonas infernus. Carbohydr Res. 2004; 339: 2371–2380. PMID: 15389352

Vandecandelerae I, Nercessian O, Segaert E, Achouak W, Mollica A, Faimali M, et al. Alteromonas genovensis sp. nov., isolated from a marine electroactive biofilm and emended description of Alteromonas macleodi. Baumann et al. 1972 (Approved Lists 1980). Int J Syst Evol Microbiol. 2008; 58: 2589–2596. doi: 10.1099/ijs.0.65691-0 PMID: 18984698

Weiss MS, Abele U, Weckesser J, Welte W, Schultz GE. Molecular architecture and electrostatic properties of a bacterial porin. Science. 1991; 254: 1627–1630. PMID: 1721242

Stoderegger K, Herndl GJ. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol Oceanogr. 1998; 42: 877–884.

Hoppe HG, Kim SJ, Gocke K. Microbial decomposition in aquatic environments: combined process of extracellular enzyme activity and substrate uptake. Appl Environ Microbiol. 1988; 54: 784–790. PMID: 16347586
39. Sutherland IW. Polysaccharases for microbial exopolysaccharides. Carbohydr Polym. 1999; 38: 319–328.

40. Liu J, Jiao N, Tang K. An experimental study on the effects of nutrient enrichment on organic carbon persistence in the western Pacific oligotrophic gyre. Biogeosciences. 2014; 11: 5115–5122.

41. Zweifel UL, Normann B, Hagstrom A. Consumption of dissolved organic carbon by marine bacteria and demand for inorganic nutrients. Mar Ecol Prog Ser. 1993; 101: 23–23.

42. Sutherland I. Biosynthesis of microbial exopolysaccharides. Adv Microb Physiol. 1982; 23: 79–150. PMID: 6180610

43. Yamashita Y, Cory RM, Nishioka J, Kuma K, Tanoue E, Jaffé R. Fluorescence characteristics of dissolved organic matter in the deep waters of the Okhotsk Sea and the northwestern North Pacific Ocean. Deep Sea Research Part II: Topical Studies in Oceanography. 2010; 57: 1478–1485.

44. Milbrandt E, Coble P, Conmy R, Martignette A, Siwicke J. Evidence for the production of marine fluorescent dissolved organic matter in coastal environments and a possible mechanism for formation and dispersion. Limnol Oceanogr. 2010; 55: 2037–2051.

45. Romera-Castillo C, Sarmento H, Álvarez-Salgado XA, Gasol JM, Marrasé C. Net production and consumption of fluorescent colored dissolved organic matter by natural bacterial assemblages growing on marine phytoplankton exudates. Appl Environ Microbiol. 2011; 77: 7490–7498. doi: 10.1128/AEM.00200-11 PMID: 21742918

46. Yamashita Y, Tanoue E. Production of bio-refractory fluorescent dissolved organic matter in the ocean interior. Nature Geoscience. 2008; 1: 579–582.

47. Chen RF, Bada JL. The fluorescence of dissolved organic matter in seawater. Mar Chem. 1992; 37: 191–221.

48. Wang Z, Gao M, Xin Y, Ma D, She Z, Wang Z, et al. Effect of C/N ratio on extracellular polymeric substances of activated sludge from an anoxic-aerobic sequencing batch reactor treating saline wastewater. Environ Technol. 2014; 35: 2821–2828. doi: 10.1080/09593330.2014.924563 PMID: 25176486

49. Kirchman DL. The ecology of Cytophaga–Flavobacteria in aquatic environments. FEMS Microbiol Ecol. 2002; 39: 91–100. doi: 10.1111/j.1574-6941.2002.tb00910.x PMID: 19709188

50. Brakstad OG, Lodeng AGG. Microbial diversity during biodegradation of crude oil in seawater from the North Sea. Microbial Ecology. 2005; 49: 94–103. PMID: 15893864

51. Li C, Du M, Cheng B, Wang L, Liu X, Ma C, et al. Close relationship of a novel Flavobacteriaceae alpha-amylase with archaeal alpha-amylases and good potentials for industrial applications. Biotechnol Biofuels. 2011; 4: 18. doi: 10.1186/1754-6834-4-18 PMID: 21485024

52. Geller A. Comparison of mechanisms enhancing biodegradability of refractory lake water constituents. Limnol Oceanogr. 1986; 31: 755–764.

53. Mann AJ, Hahnke RI, Huang S, Werner J, Xing P, Barbeyron T, et al. The genome of the alga-associated marine flavobacterium Formosa agariphila KMM 3901T reveals a broad potential for degradation of algal polysaccharides. Appl Environ Microbiol. 2013; 79: 6813–6822. doi: 10.1128/AEM.01937-13 PMID: 23995932

54. Nyvall Collén P, Sassi JF, Rogniaux H, Marfaing H, Helbert W. Ulvan lyases isolated from the Flavobacteria Persicivirga ulvanivorans are the first members of a new polysaccharide lyase family. J Biol Chem. 2011; 286: 42063–42071. doi: 10.1074/jbc.M111.271825 PMID: 22009751

55. Bakunina I, Nedashkovskaya O, Balabanova L, Zvyagintseva T, Rasskasov V, Mikhailov V. Comparative analysis of glycoside hydrolases activities from phylogenetically diverse marine bacteria of the genus Arenibacter. Mar Drugs. 2013; 11: 1977–1998. doi: 10.3390/md11061977 PMID: 23752354

56. Nedashkovskaya OI, Kim SB, Lysenko AM, Frolova GM, Mikhailov VV, Baes KS. Bizionia paragorgiae gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the soft coral Paragorgia arborea. Int J Syst Evol Microbiol. 2005; 55: 375–378. PMID: 15653903

57. Nelson CE, Carlson CA. Tracking differential incorporation of dissolved organic carbon types among diverse lineages of Sargasso Sea bacterioplankton. Environ Microbiol. 2012; 14: 1500–1516. doi: 10.1111/j.1462-2920.2012.02738.x PMID: 22937662

58. Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, et al. The genomic basis of trophic strategy in marine bacteria. Proc Natl Acad Sci USA. 2009; 106: 15527–15533. doi: 10.1073/pnas.0903607106 PMID: 19805210