Adaptation of intertidal biofilm communities is driven by metal ion and oxidative stresses

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Marine organisms in intertidal zones are subjected to periodical fluctuations and wave activities. To understand how microbes in intertidal biofilms adapt to the stresses, the microbial metagenomes of biofilms from intertidal and subtidal zones were compared. The genes responsible for resistance to metal ion and oxidative stresses were enriched in both 6-day and 12-day intertidal biofilms, including genes associated with secondary metabolism, inorganic ion transport and metabolism, signal transduction and extracellular polymeric substance metabolism. In addition, these genes were more enriched in 12-day than 6-day intertidal biofilms. We hypothesize that a complex signaling network is used for stress tolerance and propose a model illustrating the relationships between these functions and environmental metal ion concentrations and oxidative stresses. These findings show that bacteria use diverse mechanisms to adapt to intertidal zones and indicate that the community structures of intertidal biofilms are modulated by metal ion and oxidative stresses.

A biofilm is a complex aggregate of microorganisms, such as bacteria, diatoms, fungi, and protozoa, in extracellular polymeric substances (EPS) and can develop on almost all the surface1. The microbial biofilm is regarded as a preferred mode of life and is now viewed as a common adaptation, perhaps even a life stage, of most microbes in natural systems1. Microbial communities of natural biofilms in the marine environment change in response to various environmental factors, including salinity, temperature2, dissolved oxygen3, and habitat4.

In intertidal areas, which are considered as interfaces of the ocean, atmosphere, and terrestrial environments, the biofilm communities are subjected to fluctuations in metal ion concentrations, temperature, desiccation, UV irradiation, and wave activities4. The organisms in intertidal zones can spend half of their lives in extreme, arid conditions during emersion and half of their lives in stable, benign seawater. In contrast, subtidal systems are more stable because they are submerged most of the time and only exposed briefly during the extremely low tides of full and new moon events. In contrast to intertidal zones, this zone provides a habitat for a large diversity of plants and animals5–7.

Physiological adaptations are crucial for bacteria and higher organisms to adapt to changing environmental conditions and are considered to play a major role in determining which species will be the “winner” in response to global climate change. In highly variable environments such as intertidal zones, some marine organisms can utilize metabolic depression, anaerobic energy production, and stress-protective mechanisms to guard against environmental stresses6,10. Variations in biofilm components that develop at different vertical sites along the shore were detected11–13, which indicates that the structure of microbial communities in biofilm is shaped by environmental factors.

In our previous studies, we examined bacterial community succession (from 3 to 20 days) of subtidal biofilms, and biofilms of ≤9 days old demonstrated clearly different ribotypes from those of ≥12 days old14. However, the effects of tide levels on biofilms and the mechanisms employed by microbes in biofilms to cope with variations in the intertidal environment require further investigation. Metagenomic analysis is an ideal technique for addressing the issues above mentioned because it allows us to explore significant differences in metabolic potential in different environments. In the present study, we used a metagenomic analysis to identify differences in the microbial community composition and functions of 4 types of biofilms developed at 2 tidal levels (intertidal and subtidal) and for 2 different durations (6 and 12 days, defined as two different biofilm development stages by
Chung et al., 2010). We discovered that the genes responsible for tolerance to oxidative and metal ion stresses were enriched in intertidal biofilms. In addition, the genes responsible for the biosynthesis of EPS, which is important in environmental stress responses, were also enriched in intertidal biofilms. Furthermore, we characterized the taxonomic and functional differences between intertidal and subtidal biofilms and provided a suitable model to elucidate how microbes in intertidal biofilms cope with stresses in such harsh environments.

**Results**

Comparison of clusters of orthologous group (COG) categories between intertidal and subtidal biofilm metagenomes. Pyrosequencing data for the microbial metagenomes of 4 biofilm samples are summarized in Table 1. The total number of sequences ranged from 451,100 for the 12-I biofilm (hereafter referred to as 6-I and 12-I for the 6- and 12-day intertidal biofilms, and 6-S and 12-S for the 6- and 12-day subtidal biofilms, respectively), and up to 534,336 for the 6-S biofilm, with an average length of ~500 bp. Functional analysis of the biofilm metagenomes by assignment of metagenomic sequences to the COG database was performed. To identify functions with significant differences among different biofilms, a quantitative comparison of 4,223 individual COGs among these 4 biofilm samples was generated by best classification. Parallel comparison of I-12 versus S-12 and I-6 versus S-6 by STAMP (Fisher’s G-test, P < 0.01) revealed that nearly all of the significantly changed individual COGs belonging to the categories of inorganic ion transport and metabolism, secondary metabolite biosynthesis, transport and catabolism and signal transduction mechanisms were enriched in intertidal biofilms (Fig. 1). This result was consistent with the significantly changed individual COGs indicated by the binomial, hypergeometric and G-test (P < 0.01). Regarding functional diversity, all of the metagenomes displayed a similar Shannon index (6.26, 6.45, 6.47 and 6.42 for 12-I, 12-S, 6-I and 6-S, respectively).

Metal ion transporter and secondary metabolism genes. As mentioned above, genes involved in inorganic ion transport and secondary metabolism were over-represented in the intertidal metagenomes (Fig. 1). Six genes involved in inorganic ion transport and metabolism were enriched in intertidal biofilm metagenomes compared with their subtidal counterparts, including cation transport ATPases, antporters, pumps and catalase (Fig. S1). These genes are responsible for transporting a variety of ions: Ca^{2+}, Zn^{2+}, Fe^{2+}, Na^{+}, Co^{2+} and Cd^{2+}. Cation transport ATPase is a large superfamily that can utilize the energy of ATP to transport ions against electrochemical gradients and to fuel many antiporters and cation pumps. The enrichment for these transporters suggested that the microbes in intertidal biofilms adopted an energy-consuming mechanism to maintain ion homeostasis.

| Sample ID | 12-I | 12-S | 6-I | 6-S |
|-----------|------|------|-----|-----|
| No. of reads | 451,100 | 465,162 | 507,489 | 534,336 |
| Average length (bp) | 532 | 487 | 484 | 512 |
| Reads removed after quality control | 107,512 | 169,545 | 144,995 | 133,915 |
| Reads assigned to individual COGs | 173,676 | 164,814 | 239,876 | 221,765 |
| Reads assigned to KEGG EC numbers | 58,301 | 65,888 | 71,900 | 56,932 |
| Reads assigned to CAZy enzymes | 22,314 | 26,991 | 29,784 | 27,989 |

6-I and 12-I indicate 6- and 12-day intertidal biofilms, and 6-S and 12-S indicate 6- and 12-day subtidal biofilms, respectively.
The abundance of catalase genes in the 12-I biofilm metagenome. Reads of catalase (COG0735), cation transport ATPase (COG0474), thioesterase domains of type I polyketide synthases or non-ribosomal peptide synthetase (COG3319) and GT-Pase involved in the stress response (COG1217) were extracted from the 12-I biofilm metagenome and BLASTed against the NCBI-nr database and assigned to different genera using MEGAN.

Catalase is an enzyme that uses hydrogen peroxide as a substrate to produce water and oxygen. The abundance of catalase genes in 12-I biofilm was 8-fold higher than that in the 12-S biofilm (Fig. S1). Two polyketide synthase (PKS) COGs (COG3319 and COG3321) were enriched in the intertidal biofilm metagenomes. Non-ribosomal peptide synthetases (NRPSs) and PKSs are necessary to produce a variety of secondary metabolites, such as the siderophores involved in iron acquisition, to cope with intracellular oxidative stress. The 6-1 biofilm showed enrichment for anthrancene-degrading 2-hydroxyxochromene-2-carboxylate isomerase, which can induce oxidative stress in eukaryotes. All of the above-mentioned genes were related to the oxidative stress response. Figure 2 shows the taxonomic assignment of catalase (COG0735), type I polyketide synthases (COG3319), cation transport ATPase (COG0474), and a predicted membrane GT-Pase involved in the stress response (COG1217) from the 1-12 biofilm metagenome. Most of the reads for genes encoding catalase and cation transport ATPase belonged to the Proteobacteria, while type I polyketide synthases belonged to the Proteobacteria, Cyanobacteria and Firmicutes. These results suggested that Proteobacteria provided the largest contribution to the functional gene pool enriched in intertidal biofilms.

Signal transduction pathways. In addition to inorganic ion transport and secondary metabolism, another COG category enriched in intertidal biofilms was signal transduction (Fig. 1 and Fig. S3). Comparison of the 12-I biofilm with the 12-S biofilm metagenome revealed 18 signaling transduction genes with significantly differences in abundance; all of these genes were enriched in the 12-I biofilm and included several serine/threonine protein phosphatase (PSP), a GT-Pase involved in the stress response and cAMP-binding proteins, all of which regulate the stress response in bacteria. Similarly, 5 genes had a significantly higher abundance in the 6-I biofilm compared with the 6-S biofilm, including the universal stress protein UspA and related nucleotide-binding proteins, adenylate cyclase and three histidine kinases. The enrichment for these signal transduction genes in intertidal biofilms suggested that they might play a protective role against environmental stresses. The microbes with GT-Pase genes (COG1217) involved in the stress response in the 12-I biofilm are illustrated in Figure 2, indicating most of these microbes with these genes were the Proteobacteria and Bacteroidetes.

Comparison between the 12-I and 12-S biofilm metagenomes. Because genes that cope with metal ion and oxidative stress were enriched in intertidal biofilms, we tested whether the enrichment intensified during biofilm development. The 12-I and 6-I biofilm metagenomes were compared. As indicated in Figure S4, most of the COGs mentioned above displayed a significantly increased abundance in the 12-I biofilm in comparison with the 6-I biofilm, as indicated by the comparison of catalase, polyketide synthases, cation transporters and stress regulators. Consistently, a Cu/Zn superoxide dismutase (SOD) was present only in the 12-I biofilm metagenome. This enzyme is important for protection against oxidative stress. Moreover, anti-sigma factor (COG3806), which is a well-known regulator of the stress response, was also significantly enriched in the 12-I biofilm. These results indicate a community shift driven by increasing stress during the development of intertidal biofilm development.

Discussion

In this study, a metagenomic analysis was conducted to examine functional differences between intertidal and subtidal biofilms that developed for 6 and 12 days, respectively. The functional diversity of the intertidal and subtidal biofilms was similar, whereas the spectra of metabolic functions differed between them. The COG categories of secondary metabolism, inorganic ion transport and metabolism and signaling transduction were enriched in intertidal biofilms. The higher frequency of cation transport and pump-related genes, which regulate metal ion homeostasis, might reflect the elevated levels of metal ion stress faced by intertidal biofilms, which are more persistent than that of subtidal zones and subjected to salt accumulation over time. The enrichment of catalase in the 12-I biofilm metagenome indicated that intertidal biofilms might be exposed to oxidative stress. In addition, we found that the enzyme commission (EC) number of genes encoding superoxide dismutase (SOD) was significantly different from that in the subtidal biofilm. This might suggest that SOD has a role in the stress response of intertidal biofilms.

EPS biosynthesis pathways. Biofilm’s EPS, which contains several components including monosaccharides and polysaccharides, plays an important role in environmental stress responses, including the protective response against oxidative stress and ion chelation. We hypothesized that enhancement of EPS synthesis might be a strategy employed by intertidal biofilms to cope with environmental stresses. Because the results obtained for the COG categories revealed a lack of congruency among intertidal and subtidal biofilms, metagenomic sequences were assigned to the KEGG database, which is particularly useful for comparison of metabolic pathways, with the goal of identifying EPS components associated with metabolic pathways. Figure 3 illustrates the pathways involved in carbohydrate metabolism and glycan biosynthesis and metabolism that were significantly changed between intertidal and subtidal biofilms. Compared with the 12-S biofilm, all of the sugar biosynthesis pathways were enriched in the 12-I biofilm, e.g., metabolism of amino sugars, starch and sucrose, fructose and mannose and biosynthesis of N-glycan (Fig. 3A), which are common in bacterial EPS. Similarly, compared to the 6-S biofilm, biosynthesis pathways associated with fructose and mannose metabolism as well as O-glycan biosynthesis were enriched in the 6-I biofilm (Fig. 3B). In addition, a BLAST search against the Carbohydrate-Active Enzymes (CAZy) specialist database confirmed the results and revealed detailed activities of carbon metabolism proteins. Comparison of the 12-I and 12-S biofilm metagenomes using STAMP (Fisher’s G-test, P < 0.01) showed that a significant difference in all of the CAZy protein families (Fig. 4). In particular, the 12-I biofilm was enriched for polysaccharide biosynthesis gene families, including cellulose synthase, chitin synthase, xylanases and glucanase. These results suggested that enhancement of EPS might be a strategy utilized by microbes in intertidal biofilms to cope with environmental stresses.

In summary, the intertidal biofilms are exposed to a variety of environmental stresses, which are more persistent than that of subtidal zones and subjected to salt accumulation over time. The enrichment of catalase in the 12-I biofilm metagenome indicated that intertidal biofilms might be exposed to oxidative stress. In addition, we found that the enzyme commission (EC) number of genes encoding superoxide dismutase (SOD) was significantly different from that in the subtidal biofilm. This might suggest that SOD has a role in the stress response of intertidal biofilms.
Figure 3 | KEGG pathways associated with carbohydrate metabolism and glycan biosynthesis and metabolism that were significantly different between intertidal and subtidal biofilm metagenomes. Comparison of the pathways was based on the abundance of corresponding EC numbers. **P < 0.01 and ***P < 0.001. A. 12-I biofilm vs. 12-S biofilm. B. 6-I biofilm vs. 6-S biofilm.

Figure 4 | CAZy families with significant differences between the 12-I and 12-S biofilms (Fisher’s G-test, P < 0.01). **P < 0.01 and ***P < 0.001.
more abundant in the 12-I biofilm metagenome compared with the 12-S metagenome (0.08% vs. 0.05%, P < 0.05). Reactive oxygen species are commonly produced in the ocean, and oxidative stress is an important component of the stress response in marine organisms exposed to a variety of insults such as thermal stress, UV radiation, and pollution. Thus, apart from catalase, other genes that contribute to resistance to oxidative stress, such as special secondary metabolism genes, were also enriched in both 12-day and 6-day intertidal biofilms. The production of secondary metabolites is often associated with environmental stresses, including metal ion and oxidative stresses. For example, the polyketides are a large class of secondary metabolites that are present in bacteria, fungi and plants and contribute to virulence and responses to oxidative stress.

Following Cyanobacterium andabaena exposure to oxidative stress, the gene cluster encoding polyketide synthase is activated and side-ophore release is increased to acquire Fe³⁺ and enhance electron transport. The observed enrichment for these genes supported the hypothesis that oxidative stress plays an important role in shaping the functional structure and adaptability of intertidal biofilms. In fact, metal ion homeostasis and oxidative stress are considered to be coupled. For instance, iron, in its reduced form, potentiates oxygen toxicity by converting, via the Fenton reaction, the less reactive hydrogen peroxide to the more reactive oxygen species, hydroxyl radical and ferryl iron (Fe³⁺ + H₂O₂ + H⁺ → Fe⁴⁺ + O₂⁻ + H₂O). Furthermore, toxic metals such as lead, cadmium and mercury, among others, deplete the cells’ major antioxidants and increase the production of reactive oxygen species (ROS) such as hydroxyl radical (HO•), superoxide radical (O²⁻) and hydrogen peroxide (H₂O₂). The simultaneous enrichment of ion transport genes and oxidative response genes also suggests a correlation between responses to these two types of stresses.

A BLAST analysis against the KEGG database revealed that genes associated with sugar metabolism and biosynthesis (typically found in bacterial EPS) were enriched in intertidal biofilm metagenomes. This finding was further supported by results of the CAzy protein activity comparison, demonstrating enrichment of polysaccharide metabolism activity in the 12-I biofilm. For example, in enzymology, cellulose synthase [GT2] is an enzyme that catalyzes the chemical reaction UDP-glucose + (1,4-beta-D-glucosyl)n → UDP + (1,4-beta-D-glucosyl)n+1. The presence of both polysaccharide biosynthesis and degradation enzymes in the same biofilm might be attributed to the physiological heterogeneity in biofilms. The enrichment for these genes indicated that enhancement of EPS might be another strategy adopted by microbes to resist the harsh intertidal environment. Similar results were obtained in a previous study in which exposure of Pseudomonas fluorescens biofilm to copper-ion-induced oxidative stress increased biofilm-specific exopolysaccharide-related metabolism. In addition, taxonomic assignment of the genes enriched in intertidal biofilm metagenomes (Fig. 2) indicated that stress resistance in intertidal biofilms could be partly attributed to Bacteroidetes, which specialize in degrading complex organic matter such as polysaccharides and their presence also supports the hypothesis that intertidal biofilms contain abundant EPS.

Intertidal biofilms had enriched signal transduction genes. PSP is a form of phosphoprotein phosphatase that acts upon serine/threonine residues and serves as an “on-off” signal transduction switch that is triggered by environmental stresses. Members of the GTPase superfamily are extremely important in regulating membrane signalizing pathways in all cells and have been reported to regulate the formation of bacterial biofilms. Alternatively, cAMP-binding proteins can also mediate biofilm formation through environmental stress responses. It has been reported that in Escherichia coli, an increase in cellular cAMP levels results in overexpression of cattE genes, which encode catalase. The UspA domain has been detected in more than 1,000 different genes and is often fused with other domains, as exemplified in kdpD, the sensor kinase that regulates high-affinity K⁺ transport. The UspA gene family, adenylate cyclase and histidine kinase can be induced by oxidative stress. These enzymes activate downstream signaling and regulate the stress response. Therefore, in the present study, one of the underlying mechanisms of stress resistance might be the transcriptional control of genes involved in oxidative and metal ion stresses in intertidal biofilms by these signal transduction regulators.

Laboratory studies have documented extensively that the process of biofilm development is coupled with improved stress-coping ability. The results from the present study demonstrated that enrichment of genes responsible for resistance to metal ion and oxidative stresses in the 12-I biofilm in comparison with the 6-I biofilm, indicating that in response to these stresses, species sorting and a shift in community structure occurred during intertidal biofilm development. In a previous study, micro-scale differentiation was observed between the base and streamers of stream biofilms where the Proteobacteria and Bacteroidetes were strong competitors in terms of their effective responses to the environmental dynamics. In the present study, although we did not perform architectural microscopy to observe differentiation during biofilm development, our results indicated the presence of a functional shift during biofilm development in response to environmental stresses and that Proteobacteria and Bacteroidetes also contribute to the stress resistance in intertidal biofilms.

In summary, findings from the present study elucidated the stresses that shape the community structures of intertidal biofilms compared with subtidal biofilms. We hypothesize that intertidal biofilm signaling regulators are activated by metal ion and oxidative stresses present in this highly variable environment. Subsequently, the expression of EPS, secondary metabolites and ion transporters may occur (Fig. 5). Thus, the community is shaped by the two stresses, and environmental selection causes differences between intertidal and subtidal biofilm structures, as supported by the enrichment of genes responsible for coping with the two stresses in the 12-I biofilm compared with the 6-I biofilm. The pursuit of nutrients may be considered to play a more important role in shaping community structure than stresses imposed by the changing environment, as some organisms in intertidal zones suffer from nutrient limitation. However, genes encoding sugar metabolism enzymes were more strongly represented by reads in the intertidal biofilms, and energy-dependent ion transporters and pumps were highly abundant in intertidal biofilms, which indicated that energy-saving mechanisms were not used and nutrient deficiencies did not occur in intertidal biofilms. Thus, the enrichment for these sugar metabolism genes should be considered as an adaptive mechanism to environmental stress. In conclusion, adaptation of the intertidal biofilm community is produced by metal ion and oxidative stresses. There is evidence that secondary metabolites and bacterial cell-to-cell signaling mediate interactions between bacteria and eukaryotes. Therefore, in addition to stress tolerance, the enrichment for secondary metabolism and signal transduction genes might also explain why intertidal microbial biofilms are more attractive to eukaryotes for settlement. However, direct linkage between these phenomena is still lacking. Finally, because our conclusions are based on analysis of metagenomes from only one intertidal site, additional data from a broader range of sites and during different seasons are required to evaluate whether the present results occur consistently between intertidal and subtidal biofilms.

Methods

Development and collection of biofilms. Multispecies biofilms were developed on polyesterene Petri dishes in Port Shelter Bay, Hong Kong (22°19′43″N, 114°16′24″E) at 2 different tidal levels—in intertidal and subtidal—in July 2012. Petri dishes (60-mm diameter, Falcon #3002, Becton Dickinson & Co. USA) attached to large plastic panels were covered with a 125-μm mesh to prevent settlement of marine invertebrates. As mentioned in the results part, 6 and 12 days were defined as two different biofilm development stages. Thus, the panels were submerged for 6 or 12 days, resulting in the collection of 4 treatments at 2 tidal levels. After submission for 6
or 12 days, the Petri dishes were retrieved and transported back to the laboratory in a cooler with in situ seawater. The Petri dishes were washed twice using autoclaved 0.22 μm-filtered seawater (AFSW) to remove loosely attached particles and bacteria and then used for DNA extraction.

**DNA extraction and pyrosequencing.** For each tidal level and duration, biofilms developed in 40 Petri dishes were harvested using sterile cell scrapers and suspended in Tris–HCl buffer. Before nucleic acid extraction, microbial cells were pelleted by centrifugation at 4000 rpm for 10 min and then lysed with lysozyme, proteinase K and 10% SDS. Total nucleic acid was extracted and purified using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The quality and quantity of DNA were checked by agarose gel electrophoresis and with a Nanodrop device (ND-1000 spectrophotometer, DiaMed China Limited, Hong Kong) at 260 nm.

To characterize the microbial community in the biofilm samples and to determine major pathways in the different biofilms, ~3 μg of DNA was extracted from each of the 4 biofilm samples and pyrosequenced individually using the ROCHE 454 FLX Titanium platform.

**Metagenomic analysis.** Metagenomic analysis was performed as previously described13–15, Quality control for the pyrosequencing reads was conducted using the next generation sequencing (NGS) QC toolkit50. Reads <100 bp were removed, and then Ambiguity Filtering was performed to remove sequences containing 5 or more repetitive Ns before applying the CDHIT-454 to identify and discard duplicates and then Ambiguity Filtering was performed to remove sequences containing 5 or more repetitive Ns before applying the CDHIT-454 to identify and discard duplicates and then using the program “Filtering by length”, 16S rRNA fragments of <150 bp were removed. Taxonomic classification was conducted using the online RDP Classifier version 2.5 (http://rdp.cme.msu.edu) with a confidence threshold of 50% for classification at the KEGG (version 67.0)51, CAZy52 and COG (version 9.05)53 prokaryote databases on local servers, using an E-value of <10−5 to reveal the top 10 hits. Subsequently, the taxonomies were summarized using MEGAN16 and genera with an identification score >80 were retained.

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Author contributions

W.P.Z., Y.W. and P.Y.Q. wrote the main manuscript. O.O.L., Y.X., R.M.T., Z.M.G., Y.X.L. and P.Y.Q. designed and performed the experiments. W.P.Z., Y.W. and H.L.C. analyzed the data. W.P.Z., Y.W. and P.Y.Q. designed and performed the experiments. W.P.Z., Y.W. and H.L.C. analyzed the data. W.P.Z., Y.W. and P.Y.Q. prepared the manuscript. O.O.L., Y.X., R.M.T., Z.M.G., Y.X.L. and P.Y.Q. prepared the manuscript. O.O.L., Y.X., R.M.T., Z.M.G., Y.X.L. and P.Y.Q. prepared the manuscript. O.O.L., Y.X., R.M.T., Z.M.G., Y.X.L. and P.Y.Q. prepared the manuscript.

Additional information

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