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Article

Instrumental Analysis of Aquatic Organic Pollutants and Its Association with the Spread of Multi-Drug Resistant Bacteria

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Abstract: The eutrophication of open water bodies, especially in coastal water area, has received intensive public focuses in recent years. Biological treatment of organic pollutants in industrial and domestic wastewater requires microbial flora to participate. The environmental impact of these microbes, particularly in regard to the spread of antibiotic resistance genes and pathogenicity in coastal water, remains largely unknown. We initiate studies to examine antibiotic resistance genes (ARGs) in microbial flora and study the mode of antibiotic spread in the coastal waters of Bohai Bay, North China by using high performance liquid chromatography-mass spectrum analysis and quantitative polymerase chain reaction. The metabolic features of bacteria related to the presence of ARGs in the coastal area showed the enrichment of *Pseudomonadale* species with triple resistance properties (ampicillin, kanamycin and gentamycin) at the estuary site locating adjacent to a coastal sewage treatment plant where both type I and type II polyketide synthase genes and β-lactam type antibiotics are detected. *Pseudomonadale* order is phylogenetically belonged to *Proteobacteria* Phylum, *Gamma-proteobacteria* Class and contains *Pseudomonadaceae* family and *Pseudomonas* genus. QPCR analysis suggests that the percent occupation of *Pseudomonadale* species to total *Proteobacteria* in the examined stations is close to several sampling stations close to sewage treatment plants, where consistent release of organic wastes occurred.

Keywords: multi-drug resistant bacteria; microbial treatment; coastal organic waste; *Pseudomonadale*; marine microorganisms

1. Introduction

The antibiotic resistance appeared since nineteen forties after the discovery and use of penicillin-type compound in treating wounds in soldiers during World War II and has become a global concern in recent years, particularly with the appearance of multi-drug resistant bacteria. (King et al., 2014; Reardon, 2014; Woolhouse and Ward, 2013) The combined use of multiple antibiotics has become common applications and has created increasing fear that microbes obtain their resistance capacity through the swift integration and modification of resistance genes either into chromosome or plasmid to acquire higher chances to survive in antibiotic-contaminated niches. The release of antibiotics and resistance genes into the environment has inspired intensive research efforts to trace their distribution pattern and mode of degradation. (Martinez, 2009; Pruden et al., 2006; Rizzo et al., 2013) This makes the search for exact mechanism causing the occurrence of multi-drug resistant microbes becoming uncertain.

In this study, microbial composition and ARG presence in the coastal waters of Bohai Bay, North China are analyzed by examining the metabolic features related to the presence of ARGs in the coastal area by using high performance liquid chromatography-mass spectrum analysis and quantitative polymerase chain reaction. *Pseudomonadale* is found to be the main contributor to ampicillin and kanamycin resistance genes, whose population abundance is closely related to the entire

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Proteobacteria judged by using the experimental data collected at multiple geographic sites. (Young et al., 2013; Becerra-Castro et al., 2016; Sauvetre and Schroder, 2015; Whiteley and Bailey, 2000) HPLC-MS analysis to detect residual antibiotics present in coastal water samples collected in this study suggests that a few water samples contain low concentration of β-lactam antibiotics while most of the water sample collected were tested positive in either type I or type II polyketide synthase biosynthetic genes.

2. Materials and Methods

2.1. Distribution of sampling stations and collection of water samples

A total of 19 sampling stations were chosen for a general survey of bacteria-bearing ARGs, that covered the estuary sites of 11 major rivers that carry either industrial or municipal wastewater and eight additional stations extending from estuary sites to the coastal area, Qinhuangdao (N40°00, E119°54'to N39°36', E119°18') (Figure S4 (a)). Water samples (2000 mL each) from each station were collected and stored in aseptic plastic bottles in a 4℃ incubator. To collect the microbial biomass, the water sample was vacuum filtered with a microfilter of 0.22-μm pores. The filters were stored at -80℃ prior to DNA extraction and analysis.

2.2. Detection of conserved polyketide synthases and antibiotics

To trace the potential source of antibiotics within the coastal water bodies, two pairs of degenerate primers (F-DEG-PKS1/R-DEG-PKS1 (CGGGGCACCGCCATSAACMASGRCG /CGCCCAGCGGGGTGSCSGTN CCGTG) and F-DEG-PKS2/R-DEG-PKS2 (CCACCCGCTACGSSBHCCACA /CGTTCTGCTTGGTGCC GSWNCCGTGSGC)) were designed to amplify two internal fragments of the conserved type I polyketide synthase (201 bp) and type II polyketide synthase (147 bp) within the bacterial hosts. For all of these reactions, the reaction system consisted of 8 μL of sterile distilled water, 10 μL of Taq polymerase mix, 0.5 μL of the forward/reverse primers (100 mol/L), and 1 μL of DNA template in a total capacity of 20 μL. The thermal cycle was as below: initial denaturation at 95 ℃ for 5 min, then 34 cycles of in-cycle amplification with denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min; and final prolongation at 72℃ for 5 min. The PCR products were detected by agarose gel electrophoresis and analyzed by a gel imaging analyzer.

To detect the presence of the three tested antibiotics (ampicillin, kanamycin and gentamycin) in the coastal water bodies, ultra-performance liquid chromatography-mass spectrometry (HPLC-MS) combined with an antimicrobial assay was performed using seawater extracts. A 50-mL aliquot of seawater was collected from each of the 19 sampling stations (Figure S4 (a)) and extracted along the sampling line using 5mL of ethyl acetate. The extract was dried under a laminar nitrogen gas flow for 30 minutes and then re-suspended in 1 mL of methanol.

Five microliters of the methanol solution was injected to the SHIMADZU prominence LC-20AB series high performance liquid chromatography (HPLC) coupled with the Thermo LTQ Velos liquid Pro Orbitrap high-resolution mass spectrometer (HRMS). HPLC separation was performed with a YMC-Pack Pro C18 column (YMC America, Inc). A binary gradient eluent was recruited with mobile phase A of water containing 0.1% formic acid and mobile phase B of acetonitrile containing 0.1% formic acid. The elution program was as follows: 0 min, 2% B; 5 min, 2% B; 19 min, 55% B; 29 min, 98% B; 30 min, 2% B; and 35 min 2% B. The HRMS was connected to an electrospray ionization (ESI) source and operated in the positive ionization mode with a spray voltage of 3.5 kV for first-order MS with *m*/*z* scanning in the range of 200-1000 Da, and the mass resolution of 30,000. The tested antibiotics were ionized and detected as protonated molecules ($[M+H]^+$), their sodium-ionized ($[M+Na]^+$) or potassium-ionized ($[M+K]^+$) forms. Aqueous solutions of ampicillin, kanamycin, and gentamycin at different concentrations were prepared as standard solutions and used for quantitation method development. To detect ampicillin, two additional *m*/*z* values of molecular cations were included to address the possibility of its hydrolysis (by β-lactam ring-opening) and decarboxylation. An extensive search of the appropriate *m*/*z* value (with the accurate value

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determined to 0.0001) with the same HPLC retention time of each antibiotic was conducted to detect possible derivatives of the antibiotic.

3. Results

The copy numbers of each resistance gene were calculated at all sample sites according to the standard curve created by using plasmids bearing each resistant gene as templates (Supplementary Figure S1). The correlation coefficient for each resistance gene was calculated to find the association between two independent resistance gene markers (Amp^R-Kan^R, Kan^R-Gen^R, and Amp^R-Gen^R respectively) by using the experimental methods described in Supplementary Materials and Methods. The copy numbers of the ampicillin and kanamycin-resistance genes displayed a high correlation (regression r=0.7236) among individual stations, suggesting a high likelihood of the simultaneous presence of two resistance genes in one genetic island (Figure 1 (a,c,d) and Supplementary Table S1). Water samples were then analyzed for the presence of antibiotics and high throughput genetic analysis of antibiotic biosynthetic genes. The distribution characteristics of ampicillin and kanamycin resistance of various bacteria consortium in seawater are shown in the correlation heat-map (Figure 2).

From the UPLC-HRMS analysis, ampicillin was detected in station 13 (C15) in the water samples (Figure 3). A thorough examination of all the stations revealed positive amplicons of both type I and type II PKS genes with the expected size, suggesting that microbial sources of antibiotics were present (Figure 4). The presence of antibiotic biosynthetic genes and the related antibiotics suggested that the enrichment of resistant bacteria is possibly due to the inclusion of microbial species (strategically or un-purposely) with naturally conferred resistance properties where the presence of certain antibiotics may only explain the relative chemistry stability in this area. The detection of both type I and type II PKS genes also suggests that potential polyketide type compound biosynthase genes are present in water, though the product type cannot be determined soly upon the presence of these genes (clusters).

Microbial composition assay basing on the 16S rRNA sequence analysis performed with Illumina MiSeq suggests the possibility of *Pseudomonadale* species (or a similar species) being major contributors of multi-resistance genes. (Luczkiewicz et al., 2015) The statistical chart of Operational Taxonomic Unit (OTU) classification and the sample population classification tree based on GraPhlAn is shown in Figure S5(a) and (b), respectively. Average abundance of microbial species in all five stations included *Proteobacteria* (46.0%, with 42.3% *Pseudomonadales* species within the total bacteria), *Bacteroidetes* (2.2%) and *Firmicutes* (51.6%) (Figure S6 (a-d)).

Microbial composition of the estuary and coastal water samples and their microbial composition data collected from stations locating downstream to the sewage treatment plants by Hudson Bay, USA were used for comparison. The averaged abundance of major bacterial phylum includes *Proteobacteria* (73%, including 34% *Pseudomonad* species within the total bacteria), *Bacteroidetes* (20%), *Actinobacteria* (4%) and *Firmicutes* (3%), the latter two of which are shown by statistical analysis to be the roots of evolution towards *Proteobacteria* (Lake et al., 2015). The similarity in the percent compositions of *Pseudomonadale* species in total *Proteobacteria* between the two coastal areas that are geographically distant suggests that both locations are affected by a similar sewage input (Figure S4 (b-c)). Plotting the total abundance of *Pseudomonadale* species over that of *Proteobacteria* results in a regression coefficient (R^2) of 0.7356. (Figure 1 (b) and Supplementary Table S2, Figure S8)

Examination into genome sequences of *Pseudomonadale* suggested the evolutionary trace towards the fitness into ecological niches with low redox potential. We showed that wastewater treatment plants can be differentially analyzed based on the type of eutrophication matters to predict the distribution patterns of *Pseudomonadale*, the associated antibiotic resistance and pathogenicity. The capability of *Pseudomonadale* to survive in wastewater niches ensures their proliferation advantage in the area with low redox potential and becomes a reliable marker in describing the microbial composition and associated resistance-pathogenicity properties in geographic regions close to sewage treatment plants. (Hu et al., 2016; Jayaseelan et al., 2014) The proposed enrichment mechanism of *Pseudomonad* in organic rich environment and the sequential spread of resistance gene and pathogenicity factors are shown in Figure S7: (1) Oxidoreduction stress induced by the reductive chemicals in wastewater, (2) Enrich of *Pseudomonas* strains, (3) *Pseudomonas* strains surviving low

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oxidoreductive stress by using unusual phenzine respiration, (4) Biofilm formation, pathogenicity induction and antibiotic resistance through the bacerial biofilm, (5) Siderophores (iron-chelators, pathogenicity factor) and antibiotic resistance by chemical modification.

Supplementary Materials:

Author statement: Mohammad Elsheikh: Investigation, microbial genomic DNA extraction and polymerase chain reaction design, Data curation; Yunxuan Xie: High performance liquid chromatography-mass spectrum analysis, Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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