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Polycyclic aromatic hydrocarbons (PAHs) enriching antibiotic resistance genes (ARGs) in the soils☆

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ABSTRACT

The prevalence of antibiotic resistance genes (ARGs) in modern environment raises an emerging global health concern. In this study, soil samples were collected from three sites in petrochemical plant that represented different pollution levels of polycyclic aromatic hydrocarbons (PAHs). Metagenomic profiling of these soils demonstrated that ARGs in the PAHs-contaminated soils were approximately 15 times more abundant than those in the less-contaminated ones, with Proteobacterial being the preponderant phylum. Resistance profile of ARGs in the PAHs-polluted soils was characterized by the dominance of efflux pump-encoding ARGs associated with aromatic antibiotics (e.g., fluoroquinolones and acriflavine) that accounted for more than 70% of the total ARGs, which was significantly different from representative sources of ARG pollution due to wide use of antibiotics. Most of ARGs enriched in the PAHs-contaminated soils were not carried by plasmids, indicating the low possibilities of them being transferred between bacteria. Significant correlation was observed between the total abundance of ARGs and that of Proteobacteria in the soils. Proteobacteria selected by PAHs led to simultaneously enriching of ARGs carried by them in the soils. Our results suggested that PAHs could serve as one of selective stresses for greatly enriching of ARGs in the human-impacted environment.

1. Introduction

Antibiotic resistance genes (ARGs) have been recognized as an emerging environmental 'pollutant' (Pruden et al., 2006). Antibiotic resistance encoded by ARGs is popular and abundant in various matrices collected from the highly human-impacted environment, e.g., soils, water, and sediments (Chen et al., 2013a, 2013b; Knapp et al., 2010). Potential transfer of ARGs from environmental bacteria to human pathogens may seriously impair the efficacy of antibiotic therapy, thereby posing a deleterious health risk to the public (Pehrsson et al., 2016). In some clinical cases, some antibiotics are out of use due to the prevalence of their resistance and treatment options for certain pathogens have been becoming very limited (Wenzel and Edmond, 2000), whereas bacterial infections still annually claim hundred thousands of lives worldwide (Nathan, 2004).

The soils are considered as the largest reservoir of antibiotic resistant bacteria (ARB) and ARGs (Nesme and Simonet, 2015). A large variety of novel ARGs were frequently discovered in the soils and represent all major resistance mechanisms, which largely expanded our knowledge on antibiotic resistome in the environment (Forsberg et al., 2014). Analysis of archived soils demonstrated that ARG abundance was substantially increased since antibiotic era began at 1940 (Knapp et al., 2010). However, the question is that what type of anthropogenic contaminant can result in the dissemination and accumulation of ARGs in the soils. First of all, a huge amount of commonly-used antibiotics in human

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medicine, husbandry and aquaculture establish the major human impacts on the spread and enrichment of antibiotic resistance in various environment niches (Chee-Sanford et al., 2009; Larson, 2007). Meanwhile, other contaminants with significant antimicrobial activities could not be ignored in shaping bacterial communities and accordingly selecting antibiotic resistance. Growing evidence has demonstrated the selection of ARB and ARGs by heavy metals (Baker-Austin et al., 2006), as well as by some organic contaminants, e.g., polychlorinated biphenyls (Lo Giudice et al., 2013), pesticides (Anjum and Krakat, 2016), and disinfectants (Karatzas et al., 2007). It is well believed that more organic contaminates will be found to have significant selection for the ARB and ARGs.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in various environments (Bosch et al., 2015; Choi et al., 2013), and their concentrations are unexpectedly high in some pollution accidents, e.g., oil spills and coking wastewater (Brandt et al., 2002; Wang et al., 2014; Zhang et al., 2012). As a result, community structure of indigenous bacteria in the soils was considerably changed due to the contamination of PAHs (de Menezes et al., 2012; Yang et al., 2014; Zhang et al., 2010). PAHs-tolerant bacterial isolates from the highly-impacted environment often exhibited strong resistance to metals and antibiotics as well (Ben Said et al., 2008a; Mathe et al., 2012). However, it is unknown how many types of ARGs could be enriched in the soils by the PAHs. The ongoing rapid development of sequencing-based descriptive metagenomic approaches is providing the means to analyze the occurrence and abundance of enormous well-recognized ARGs in the soils that could not be solved previously by culture-based strategies, conventional PCR technique specific to several ARGs, and combination of them (Li et al., 2015b; Monier et al., 2011). Metagenomic profiling of ARGs in the PAHs-contaminated soils could help to better understand the emergence and diversity of ARGs under PAH stresses. Moreover, ARGs could be amplified in the environment under elevated selective stress via two distinct pathways, *viz.* the overgrowth of ARGs-carrying indigenous bacteria, or horizontal gene transfer of ARGs between bacteria using mobile gene elements (MGEs) as the carriers (e.g., plasmids, transposons and integrons) (Engelstadter et al., 2016; Knapp et al., 2008; Wang et al., 2015). Therefore, analysis of bacterial community and identification of genetic location of ARGs could be beneficial to gain more insights about how ARGs are accumulated in the PAHs-contaminated soils.

The overall objective of the present study was to delineate comprehensive profile of ARGs (that is to say, the diversity and relative abundance of ARGs) enriched in the PAHs-contaminated soils using the metagenomic method. Further, analysis of the bacterial community structure and genetic locations of ARGs in the PAHs-contaminated soils were carried out to elucidate the underlying mechanisms for ARGs being enriched in the PAHs-contaminated soils via the changes of bacterial community or alternatively via horizontal transfer of genes between bacterial hosts.

2. Material and methods

2.1. Study area and sampling

Soil samples were collected from a petrochemical plant located at Guangzhou, China in July 2015 (N23.123, E113.4771). These samples were taken from three regions that represented different contamination degree of PAHs (the garden at the front gate (Site P1), tank storage area (Site P2), and wastewater pond (Site P3)). At each of sampling regions, the top 5-cm layer of soils were taken from 5 different positions using a sampling shovel, and were thoroughly mixed to avoid heterogeneous difference caused by a single time of sampling. All soil samples were immediately stored in sterilized polyethylene plastic bags and delivered back to the lab. A part of fresh soils collected at Site P3 was used to screen and isolate culturable bacterial strains with high tolerance to PAHs because this site was the most polluted by PAHs among the three sampling sites. The rest samples were frozen at −20 °C until DNA extraction and analysis of PAHs.

In the present study, an active sludge sample was collected from a sewage treatment plant in Shatin, Hong Kong in July 2007 (Yang et al., 2013), a swine feces sample was taken from a 7-8 month-old pig in a swine feedlot in Zhuhai, China in January 2013 (Li et al., 2015b), and a sediment sample was collected from an aquaculture pond in Shantou, China in December 2015. The above three samples that represented typical pollution sources of ARGs due to wide use of anthropogenic antibiotics were used as the comparisons with the soils collected from petrochemical plant. Moreover, sequencing datasets of six soil samples contaminated with oil spills were downloaded from the MG-RAST (Table S1) (Supplementary material).

2.2. Analysis of PAHs

Sixty PAHs in the soils were analyzed using the method reported in our previous study (Yuan et al., 2015). Briefly, PAHs were extracted from the soils using accelerated solvent extraction (ASE 350, Dionex) according to US EPA 3545a procedure. Approximately 10 g of dry and ground soils were mixed with certain diatomaceous earth, and then added into extraction cells. The m-Terphenoyl (200 mL, 1.0 mg L^{-1}), as surrogate standard, was spiked into each of samples before PAH extraction. Extraction cells were heated to 100 °C, pressurized with dichloromethane and acetone mixture (1:1, v/v), and maintained for 10 min. Subsequently, the extracts were concentrated using a rotary evaporator. The final volume was adjusted to 1.0 mL with acetone, and then internal standards (fluoranthene-d10, pyrene-d10, and chrysene-d12) were spiked at the concentration of 0.1 mg g^{-1}. Finally, the concentrations of PAHs were determined using gas chromatography coupled with mass spectrometry (GC-MS) in electron impact mode.

The selected ions, limit of detections (LOD), limit of quantification (LOQ), recoveries and relative standard deviation (RSD) of PAHs were presented in Table S2. The recoveries of PAHs were in the range of 64.2%–132.3%. The RSD of all PAHs was lower than 12.9%, with an exception for 2,3 + 1,4-Dimethyl naphthalene (24.8%). The limit of quantification (LOQ) with a signal-to-noise ratio (S/N) of 10 was ranged from 0.03 to 5.83 ng g^{-1}.

2.3. Isolation of culturable bacteria from the PAHs-contaminated soils

Isolation of PAHs-tolerant bacteria from the soils was carried out according to the following procedure. The soils collected from Site P3 were chosen for isolation of bacteria because this site was the most polluted by PAHs among all three sites. Approximately 5 g of soils collected from Site P3 nearby wastewater ponds were added into 50 mL of sterile sodium pyrophosphate solution (2.8 g L^{-1}), and resulted solution was shaken overnight on a horizontal rotator with 150 rpm at 28 °C. An 5-mL aliquot of bacterial culture was mixed with 45 mL minimal salt medium (MSM) ($(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CaCl_2$, 0.1 g; $K_2HPO_4 \cdot 3H_2O$ 8.22 g and KH_2PO_4 1.91 g *per* L, pH 7.2) containing 100 mg L^{-1} of 1-methyl phenanthrene (1-MP). Bacterial culture (0.5 mL) was inoculated to 50 mL of fresh MSM with 100 mg L^{-1} of 1-MP every 2 weeks. After the above inoculation was repeated 7 times, culturable bacterial consortium from the soils of Site P3 was obtained.

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