Environment International 117 (2018) 186–195

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Exploring abundance, diversity and variation of a widespread antibiotic resistance gene in wastewater treatment plants

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ARTICLE INFO

Keywords:
16S rRNA
sul1
Degenerate primer design
Quantitative PCR
MiSeq sequencing

ABSTRACT

An updated *sul1* gene sequence database was constructed and new degenerate primers were designed to better investigate the abundance, diversity, and variation of a ubiquitous antibiotic resistance gene, *sul1*, with PCR-based methods in activated sludge from wastewater treatment plants (WWTPs). The newly designed degenerate primers showed high specificity and higher coverage in both *in-silico* evaluations and activated sludge samples compared to previous *sul1* primers. Using the new primers, the abundance and diversity of *sul1* gene, together with 16S rRNA gene, in activated sludge from five WWTPs in summer and winter were determined by quantitative PCR and MiSeq sequencing. The *sul1* gene was found to be prevalent and displayed a comparable abundance (0.081 copies per bacterial cell in average) to the total bacteria across all samples. However, compared to the significant seasonal and geographical divergences in the quantity and diversity of bacterial communities in WWTPs, there were no significant seasonal or geographical variations of representative clusters of *sul1* gene in most cases. Additionally, the representative *sul1* clusters showed fairly close phylogeny and there was no obvious correlation between *sul1* gene and the dominant bacterial genera, as well as the *int1* gene, suggesting that bacterial hosts of *sul1* gene is not stable, the *sul1* gene may be carried by mobile genetic elements, sometimes integrated with class 1 integrons and sometimes not. Thus mobile genetic elements likely play a greater role than specific microbial taxa in determining the composition of *sul1* gene in WWTPs.

1. Introduction

Antibiotic resistance is one of the greatest threats to public health (Oberlé et al., 2012), with wastewater treatment plants (WWTPs) acting as both important recipients and reservoirs of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in urban environments. The activated sludge within WWTPs, containing diverse microbial species at high quantities and playing important roles in the biological treatment of sewage, is believed as ideal conditions for horizontal gene transfer (HGT) of ARGs amidst those highly active species (Munck et al., 2015; Yang et al., 2013). As antibiotics have been introduced into WWTPs *via* influent streams (Zhang and Zhang, 2011), the microbial communities present in these systems have responded to the high selection pressure by becoming enriched in ARB with ARGs (Zhang et al., 2016).

HGT is generally mediated by mobile genetic elements (MGEs), such as plasmids, transposons, and phages (Leplae et al., 2010). These MGEs could play an important role in the acquisition, expression, and dissemination of ARGs (Bennett, 2009). The sulfonamide resistance gene *sul1*, is one of the most widely spread ARGs with high abundance in various environments (Colomer-Lluch et al., 2014; Miller et al., 2016; Muziasari et al., 2017). As it is almost exclusively found on a wide range of conjugative plasmids (Heuer et al., 2012; Popowska and Krawczyk-Balska, 2013; Wu et al., 2010), the *sul1* gene should be one of the representative ARGs that closely related to MGEs. Additionally, *sul1* gene was regarded as naturally occurring in bacterial fractions of raw

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https://doi.org/10.1016/j.envint.2018.05.009
Received 16 February 2018; Received in revised form 24 April 2018; Accepted 3 May 2018

wastewater, hence it is also proper to assess the persistence of ARGs under different conditions (Calero-Caceres and Muniesa, 2016). Previous investigations indicated the associations of *sul1* gene with other ARGs (Hu et al., 2016), like aminoglycoside (Rosengren et al., 2009), beta-lactamase (Devanga Ragupathi et al., 2016), chloramphenicol (Gow et al., 2008), tetracycline (Boerlin et al., 2005; Sunde and Norström, 2006), and trimethoprim resistance genes (Hu et al., 2011). Therefore, *sul1* gene could be a good candidate to estimate the occurrence of antibiotic resistance.

A number of molecular methods have been used to detect and quantify the occurrence of ARGs in environmental samples with traditional PCR and quantitative PCR (qPCR) among the most commonly used approaches (Mu et al., 2015), as exemplified by studies on the abundance of quinolone resistance genes in wetlands (Cummings et al., 2011), chloramphenicol resistance genes in wastewater (Chen et al., 2016), sulfonamide resistance genes and tetracycline resistance genes in soils (Tang et al., 2015), sediments (Yang et al., 2016), and fresh waters (Xiong et al., 2015). In the meantime, high-throughput microarray (Sun et al., 2014; Zhang et al., 2013), high-throughput quantitative PCR (Su et al., 2015; Wang et al., 2014) and metagenomics (Yang et al., 2013) have also been gradually adopted to detect the ARGs in environmental samples, and are promising methods that may provide more comprehensive information for multiple ARGs. However, with the exception of metagenomic sequencing, the accuracy of all other PCR-based approaches largely depends on the adopted primers. Many primers used for amplification of ARGs were designed ten years earlier and have not undergone a rigorous re-assessment even though the number of available sequences has expanded tremendously in the intervening time. Minor biases in primers could excessively alter the pool of amplicons from environmental samples, thereby result in imprecise conclusions (Thomas et al., 2011). For instance, Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009), the most widely used sequence database for ARGs including *sul1*, was last updated in 2009 but is still used for the design and validation of many primers. Therefore, a more comprehensive database containing all known *sul1* gene sequences is urgently needed.

Microbial communities are known to exhibit seasonal and geographical variations in different environments (Durrer et al., 2017; Turki et al., 2017; Wang et al., 2016). For example, bacterial communities in biofilms and wastewater of WWTPs obtained in summer were found to be more abundant, complex, and variable than in winter, indicating the fluctuation of bacterial community structure existed between seasons in municipal wastewater treatment process (Turki et al., 2017). Geographical distance also generally plays a significant role in shifting the bacterial communities in WWTPs (Wang et al., 2016). For instance, the bacterial communities showed significant alterations across 26 biological WWTPs in China, and those alterations had close correlations with geographical distance (Wang et al., 2016). Accordingly, the quantities and diversities of ARGs could show dynamics and regional specificity similar to those seen in the larger bacterial community. In the present study, we chose the representative marker of antibiotic resistance, *sul1* gene, employing qPCR and MiSeq amplicon sequencing to explore the distribution of both *sul1* and 16S rRNA genes in activated sludge from WWTPs. We sought to address the two following questions. (1) Do the quantities and diversities of *sul1* gene show the geographical and seasonal changes in WWTPs like microbial communities? (2) Is the change of *sul1* gene associated with any specific bacterial taxa that could be its potential host? To answer these questions, we collected all currently available *sul1* gene sequences from public databases and designed a new pair of universal degenerate primers in order to achieve higher specificity and coverage of *sul1* gene compared to other primers in environmental samples. Based on the new primers, we further explored the abundance, diversity, existing status and phylogenetic structure of the representative ARG, *sul1*, to help illuminate the level of antibiotic resistance and dissemination mechanism of ARGs in WWTPs.

2. Methods

2.1. Sample collection

Five urban sewage WWTPs from three Chinese cities including one from Beijing (BJ), two from Qingdao (QDN, QDS) and two from Wuxi (WXN, WXS), were sampled for this study. Detailed operational parameters of the WWTPs are summarized in Table S1. Activated sludge was sampled in triplicate from aerobic tank in secondary treatment in both December 2014 and June 2015. The samples in those two seasons were designated as BJ6 (June samples), BJ12 (December samples), QDN6, QDN12, QDS6, QDS12, WXN6, WXN12, WXS6 and WXS12. Wastewater parameters, including dissolved oxygen (DO), pH, temperature, and operational parameters including inflow and effluent of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) were measured according to the Standard Method of Water and Wastewater Monitoring (Bureau, 2002) in triplicate and average values were listed in Table S2.

2.2. In silico design and assessment of sul1-targeted primers

For the purpose of selecting preferable primer pairs for the amplification of *sul1* gene, commonly used *sul1*-targeted primer pairs were collected and evaluated. At the same time, 323 non-redundant nucleotide sequences of the *sul1* gene were downloaded as *sul1*-ARDB according to the protein sequences in ARDB (Liu and Pop, 2009), and their corresponding protein sequences were aligned by ClustalW with default parameters in MEGA 6.0 (Thompson et al., 1994). HMMER 3.0 program was used to build the hidden Markov model (HMM) and search the GenBank protein database (as available on Dec 30, 2016). The obtained sequences were screened according to the sequence annotations and HMMER e-values, such that only protein sequences clearly annotated as "*sul1*" or "sulfonamide resistance" with e-values $< 10^{-2}$ were kept. The corresponding nucleotide sequences were downloaded and integrated into an updated *sul1* gene sequence database. Thereafter, in order to design a new pair of *sul1* primers with higher coverage, the DegePrime program (Hugerth et al., 2014) with the superiority of finding a degenerate oligomer of as high coverage as possible, was adopted to design degenerate primers with the aligned *sul1* nucleotide sequences. Primer coverage was determined by BLASTn against both *sul1*-ARDB and the updated *sul1* gene sequence database (Table S3). After comparison of the primers' coverage, the new primer pairs 51F (5′-AAATGCTGCGAGTYGGMKCA-3′) and 280R (5′-AACMACCAKCCTRCAGTCCG-3′) were reserved. In order to test the primer specificity, 21,176 nucleotide sequences of all ARGs were downloaded from ARDB. Based on the updated sequence database of *sul1* gene and ARDB, the specificity of 51F and 280R was assessed locally by searching the primer sequences against these databases with MFEprimer program (Qu et al., 2012) to exclude the false positive to other ARGs. Moreover, the oligonucleotide properties of 51F and 280R were further calculated by OligoCalc (Kibbe, 2007) to exclude potential hairpin formation and self-annealing.

2.3. DNA extraction and quantification of sul1, int1 and 16S rRNA genes by qPCR

Six DNA samples were extracted from 0.25 gram sediment (wet weight) of each WWTP in each sampling season (3 biological replicates × 2 experimental replicates) with a FastDNA SPIN kit for soil (Qbiogene, Solon OH). Quantitative PCR was performed to determine the abundance of *sul1* gene, *int1* gene, and bacterial 16S rRNA gene in total DNA samples with SYBR Green method. The commonly used primer sets *sul1*_1, *sul1*_2, *sul1*_3 and *sul1*_4 with relatively higher coverage (Table S3), as well as 51F and 280R were tested in activated sludge samples from Beijing WWTP. After this parallel comparison, only 51F and 280R were chosen for the quantification of *sul1* gene in all