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Metagenomic and metatranscriptomic analyses reveal activity and hosts of antibiotic resistance genes in activated sludge

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ABSTRACT

Wastewater treatment plants (WWTPs) are a source and reservoir for subsequent spread of various antibiotic resistance genes (ARGs). However, little is known about the activity and hosts of ARGs in WWTPs. Here, we utilized both metagenomic and metatranscriptomic approaches to comprehensively reveal the diversity, abundance, expression and hosts of ARGs in activated sludge (AS) from three conventional WWTPs in Taiwan. Based on deep sequencing data and a custom-made ARG database, a total of 360 ARGs associated with 24 classes of antibiotics were identified from the three AS metagenomes, with an abundance range of 7.06×10^{-1}–1.20×10^{-4} copies of ARG/copy of 16S rRNA gene. Differential coverage binning analysis revealed that > 22 bacterial phyla were the putative hosts of the identified ARGs. Surprisingly, genus *Mycobacterium* and family *Burkholderiaceae* were observed as multi-drug resistant harboring 14 and 50 ARGs. Metatranscriptome analysis showed 65.8% of the identified ARGs were being expressed, highlighting that ARGs were not only present, but also transcriptionally active in AS. Remarkably, 110 identified ARGs were annotated as plasmid-associated and displayed a close to two-fold increased likelihood of being transcriptionally expressed compared to those ARGs found exclusively within bacterial chromosomes. Further analysis showed the transcript abundance of aminoglycoside, sulfonamide, and tetracycline resistance genes was mainly contributed by plasmid-borne ARGs. Our approach allowed us to specifically link ARGs to their transcripts and genetic context, providing a comprehensive insight into the prevalence, expression and hosts of ARGs in AS. Overall, results of this study enhance our understanding of the distribution and dissemination of ARGs in WWTPs, which benefits environmental risk assessment and management of ARB and ARGs.

1. Introduction

Due to the worldwide health impacts of antibiotic resistant pathogens, the role of wastewater treatment plants (WWTPs) as a sink and source for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) is of high significance (Voolaid et al., 2017). WWTPs are generally considered as a unique interface between human society and the environment since sewage contains bacteria of both human and environmental origins as well as various antibiotic residues from households, hospitals and small-scale pharmaceutical industries (Nnadozie et al., 2017). They exercise a selective pressure for ARB and ARGs prior to the discharge into the environment (Karkman et al., 2017; Martinez, 2009). Furthermore, the presence of antibiotics in combination with high bacterial density and nutrient availability in WWTP provides suitable conditions to facilitate a high rate of horizontal gene transfer among environmental bacteria and human pathogens (Aminov, 2011; Rizzo et al., 2013; Watkinson et al., 2007). The activated sludge (AS) process is widely used for nutrient removal from wastewater in WWTPs. In this process, many ARGs associated with antibiotics, for example sulfonamide, beta-lactam, tetracycline, and vancomycin, can be enriched during digestion (Bengtsson-Palme et al., 2016; Karkman et al., 2016; Yang et al., 2014). Consequently, AS might

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play an important role in the selective enrichment and then spread of ARGs. The growing demand to reuse AS for removing nutrients poses a risk of further dissemination of antibiotic resistance in the environment. Given the potential impacts of ARB on human and animal health, there is a pressing need to conduct comprehensive investigations of prevalence and hosts of ARGs in WWTPs to support robust risk assessment and management of ARB and ARGs in wastewater impacted environments.

Previously, molecular techniques, such as polymerase chain reaction (PCR), quantitative PCR (qPCR) and DNA microarray were applied to investigate the occurrence and abundance of ARGs in WWTPs (Ben et al., 2017; Mao et al., 2015; Rodriguez-Mozaz et al., 2015; Su et al., 2015; Sun et al., 2014). However, owing to the limited availability of primers in these amplification-based approaches, little is known on the comprehensive ARG profiles in WWTPs (Yang et al., 2013). Meanwhile, metagenomic analysis can overcome these drawbacks and has been gradually adopted to reveal the broad-spectrum profile of ARGs in diverse environments (Chen et al., 2013; Guo et al., 2017; Hu et al., 2013; Li et al., 2015b; Nesme et al., 2014; Pal et al., 2016; Van Goethem et al., 2018). Nevertheless, most of these studies based on metagenomics used short reads for ARG annotation, which captures data on occurrence and abundance exclusively. Additionally assembling short reads to much longer scaffolds can provide further information about phylogeny and genetic context of these identified ARGs. In most environments, ARGs are rare in comparison to other functional genes, therefore, deep sequencing is needed to reveal their whole diversity (Bengtsson-Palme et al., 2016; Yang et al., 2013). Furthermore, deep sequencing allows reconstruction of partial or even complete genomes of ARG hosts and plasmids from metagenome data (Albertsen et al., 2013; Hultman et al., 2015; Zhang et al., 2011), which allows to detect the ARGs genomic context and establish the relationship between ARGs and their hosts. Consequently, unprecedentedly deep sequencing data was used in this study to comprehensively investigate the prevalence and hosts of ARGs in WWTP AS.

Due to the technical difficulties associated with processing of RNA samples, very few studies have focused on gene expression of ARGs in environmental samples (Ju et al., 2018; Rowe et al., 2017; Versluis et al., 2015), even though gene expression remains a far better proxy for functional activity than gene contents (Sorek and Cossart, 2010). To reach beyond the presence and absence of ARGs and gain a more complete understanding of ARG dynamics, it is necessary to reveal which of the detected ARGs are subsequently expressed. The mobility of ARGs depends on the mobile genetic elements, such as plasmids. Several studies have been successful in characterizing the diversity of ARGs encoded by plasmids by using culture-independent metagenomic approaches (Li et al., 2015a; Parsley et al., 2010; Zhang et al., 2011). However, the expression and contribution of plasmid-associated ARGs in conferring resistance to antibiotics are still largely unknown. Metatranscriptome analysis in combination with genetic context and host identification could thus extend our understanding of the risks that AS-borne ARGs pose through the augmentation of the environmental resistome. Results obtained through this type of analysis might thus be crucial for future ranking of risks associated with ARGs in environmental samples.

By employing a combination of metagenomics and metatranscriptomics it becomes possible to correlate the ARGs to their hosts and detect if either the host bacterium or the ARGs genomic context (chromosomal vs. plasmid encoded) has an effect on ARG expression. To reveal the distribution and expression of ARGs in bacterial taxa, we first examined the occurrence, abundance and diversity of ARGs in AS collected from three full-scale WWTPs using a metagenomic assembly approach. Subsequently, metatranscriptomics was employed to detect ARG transcripts and correlated them to the corresponding ARGs. Finally, to reveal which microorganisms were carrying the resistance genes, the ARG-carrying draft genomes were reconstructed and annotated to establish the relationship between ARGs and their hosts. To test if genomic context indeed plays a role in ARG expression, we assessed the contribution of plasmid-associated ARGs to the detected total and expressed ARGs. To the best of our knowledge, this is the first study combining metagenomics and metatranscriptomics data to comprehensively investigate influence of hosts and genomic context on the expression of ARGs.

2. Materials and methods

2.1. Sample collection

Three conventional WWTPs, namely BL, LK and WS located in Taiwan, were selected for sampling, and detailed information of environmental and operational parameters of these WWTPs are summarized in Table S1. The sources of the wastewater in the BL plant include landfill (86%), condensate buried (5%), and humus buried (9%) leachates. In the LK plant, the influent consisted of 100% municipal wastewater. For the third plant WS, the influent was composed of car washing wastewater (60%), landfill leachate (10%), and supernatant of kitchen waste compost (30%). Two activated sludge (AS) samples from BL and WS plants were collected from aeration tanks (Figs. S1 and S2), while the AS sample from LK plant was collected from an anaerobic tank (Fig. S3). The collection of samples from different types of WWTPs allows us to reveal the AS antibiotic resistomes associated with the different wastewater sources and treatment processes. AS samples were collected and put into sterile test tubes, placed on ice for transport to the laboratory, and then stored at −80 °C before DNA extraction. Samples for RNA isolation were immediately mixed with RNAlater (MO-BIO, USA) on site and RNA extraction was carried out after transported back into laboratory.

2.2. DNA and RNA extraction

AS samples were centrifuged at 5000 rpm for 10 min at 4 °C to remove the supernatant. Then total DNA and RNA were extracted using the PowerMax® Soil DNA Isolation Kit (MO-BIO, USA) and PowerSoil® Total RNA Isolation Kit (MO-BIO, USA), respectively. In order to obtain pure RNA, genomic DNA was removed from total RNA using the TURBO DNA-free kit (Ambion, USA) and then concentrated using the RNeasy MinElute Cleanup Kit (Qiagen, Germany). The concentrated RNA was converted to double-stranded cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA) with priming via random hexamers for first-stranded synthesis, and the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen, USA) for second-stranded synthesis. The concentration and purity of total DNA and synthetic double-stranded cDNA were checked with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). All above kits were used according to the manufacturer's instructions.

2.3. Metagenomic sequencing, assembly and binning

Metagenomic sequencing was performed at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Approximately 5 μg of DNA or synthetic double-stranded cDNA was used to construct 350 bp paired-end sequencing libraries. Paired-end sequencing was performed using Illumina HiSeq PE150 platform according to the manufacturer's instructions (Illumina, San Diego, CA). The raw metagenomic reads were dereplicated (100% identity over 100% length) using a self-written python script and trimmed using Sickle (https://github.com/najoshi/sickle) with default parameters. During the trimming step, low quality nucleotides and any read with any number of ambiguous base calls were discarded. Subsequently, trimmed reads were paired by a self-written python script and used for assembly. Whole genome de novo assemblies were performed using IDBA-UD (Peng et al., 2012) with the following parameters: -mink 65, -maxk 145, -step 10, -pre_correction. The open reading frames (ORFs) within

assembled scaffolds were predicted using Prodigal v2.6.3 (Hyatt et al., 2010).

Binning of assembled metagenomic sequences was performed using MaxBin (Wu et al., 2015) and MetaBAT (Kang et al., 2015) based on tetranucleotide frequencies, scaffold coverage and %GC content. Then the best resulting bins were chosen using DASTool (Sieber et al., 2018). Bin completeness and contamination were calculated using CheckM v1.0.6 (Parks et al., 2015) based on lineage-specific conserved marker gene sets in each genome.

2.4. Identification of ARGs and plasmid-associated genes

The completeness and correct annotation of databases are the key factors affecting the accuracy of ARG analysis. Here, three well-accepted databases of ARGs were downloaded from the Antibiotic Resistance Genes Database (ARDB, version 1.1) (Liu and Pop, 2008), Comprehensive Antibiotic Resistance Database (CARD, version 1.1.7) (McArthur et al., 2013), and ResFinder Database (ResFinder 2.1) (Zankari et al., 2012) and combined. By removing redundant and incorrect sequences, and adding a number of ARGs collected from NCBI's non-redundant (nr) protein database, an Integrated Antibiotic Resistance Genes Database (IARDB) was constructed locally. As shown in Table S2, our IARDB contains 26 antibiotic classes, 1237 ARGs, and 24,782 non-redundant reference sequences.

The protein sequences of these assembled ORFs were searched for ARGs against the IARDB developed by our group using BLASTP with an E-value $\leq 10^{-10}$ (Hu et al., 2013). An ORF was designated an ARG-like sequence if its best BLASTP alignment to ARG reference sequences was at least 60% similar to a query coverage of at least 70%. The ARG-like sequences with ≥80% sequence similarity were directly identified as ARGs (Hu et al., 2013; Ma et al., 2015). The remaining ARG-like sequences (with sequence similarity between 60% and 80%) were further confirmed by comparing against the NCBI nr protein database, and only those that had top hits to proteins with conferred resistance to related antibiotics were considered.

To detect the genetic context of ARG-carrying scaffolds (ACSs), ORFs of the scaffolds which carried identified ARGs were further compared against the ACLAME database (Leplae et al., 2009) of mobile genetic elements using BLASTP with a cutoff E-value of $\leq 10^{-10}$. The BLASTP hit outputs were filtered to annotate plasmid-associated genes using strict criteria with amino acid identity ≥80% and coverage ≥70%. If the ORFs of an ACS were annotated as plasmid-associated genes, the ACS was identified as a plasmid-associated scaffold, and the ARGs within the scaffold were identified as plasmid-associated ARGs.

2.5. Abundance of ARGs and ARG transcripts

The relative abundance of each ARG was expressed as the 'copies of ARG per copy of 16S rRNA gene' (thereafter called 'ratio'), which is similar to quantitative PCR results reported in most previous literature. ARG abundance was calculated using the following equation (Li et al., 2015b):

$$\text{Abundance(ratio)} = \sum_{1}^{n} \frac{\mathbf{N}_{\text{ARG mapped reads}} \times \mathbf{L}_{\text{reads}}/\mathbf{L}_{\text{ARG sequence}}}{\mathbf{N}_{\text{16S mapped reads}} \times \mathbf{L}_{\text{reads}}/\mathbf{L}_{\text{16S sequence}}}$$

where $N_{ARG\ mapped\ reads}$ is the number of mapped reads for each identified ARG sequence, which was determined by mapping the raw metagenomic reads to the ARG sequence using BWA with default settings (Li and Durbin, 2009); $L_{ARG\ sequence}$ is the sequence length of the identified ARG sequence; $N_{16S\ mapped\ reads}$ is the number of mapped reads for total 16S rRNA gene sequences, the 16S rRNA gene sequence was identified from the assembled scaffolds by BLASTN search against the SILVA SSU Ref database (release 128); $L_{16S\ sequence}$ is the sequence length of the 16S rRNA gene sequence; n is the number of identified ARG sequences belonging to the same antibiotic class or ARG type; L_{reads} is the sequence length of the Illumina reads (150 bp in this study).

To evaluate the expression levels of identified ARGs, metatranscriptomic reads were mapped against each ARG sequence identified from metagenomic assembly scaffolds using BWA with default settings. The abundance of a particular ARG transcript was calculated by summing the coverages (in units of 'times per Giga base', ×/Gb) of the identified ARG sequences belonging to the same antibiotic class or ARG type in each metatranscriptomic dataset. In other words, the abundance of ARG transcripts was normalized to the size of each non-rRNA data set (Gb) instead of copies of total 16S rRNA gene sequences.

$$\text{Abundance}(\times/\text{Gb}) = \sum_{1}^{n} \frac{\mathbf{N}_{\text{ARG mapped reads}} \times \mathbf{L}_{\text{reads}}/\mathbf{L}_{\text{ARG sequence}}}{\mathbf{S}}$$

where $N_{ARG\ mapped\ reads}$ is the number of mapped metatranscriptomic reads for each identified ARG; S is the size of each non-rRNA metatranscriptomic data set (Gb). The rRNA reads were identified and removed from metatranscriptome using RiboPicker (Schmieder et al., 2011). By removing rRNA reads, about 3.8 Gb, 3.9 Gb, and 8.0 Gb non-rRNA metatranscriptomic reads were filtered from BL, LK, and WS raw reads, respectively (Table S3). L_{reads}, $L_{ARG\ sequence}$ and n were the same as above.

2.6. Taxonomy annotation of ARG-carrying genome (ACG) bins

In order to ensure the accuracy for the taxonomic annotation of the ACSs, only those ACSs with a length of ≥1500 bp were considered. To further obtain the most reliable taxonomic annotation and lowest possible taxonomic level of ACSs, we selected the high quality genome bins (contamination ≤10%) that contained ACS (s), and then these ACG bins, instead of ACSs, were used to reveal the taxonomy of the ARG hosts. Taxonomic classification of the ACG bins was performed with the 'scaffold_stats', 'call_genes' and 'taxon_profile' methods of RefineM v.0.0.22 (https://github.com/dparks1134/RefineM) using default parameters based on the average amino acid identity (AAI) and shared gene content against GTDB_r80 database (Parks et al., 2017).

2.7. Statistical analysis

All statistical tests were considered significant at $p < 0.05$. The variation in microbial and ARG composition among the three AS samples was tested by Kruskal-Wallis analysis of variance (ANOVA) using PAleontological STatistics (PAST) software (version 3.16). Pearson correlation analysis was performed using PAST to identify correlations between ARG abundance and expression. Circos plot analysis was performed in the R environment using the RCircos package (Zhang et al., 2013). The ARG sequences for neighbor-joining phylogenetic trees were aligned using MAFFT v7.123b (Katoh and Standley, 2013). The trees were constructed using IQ-TREE version 1.6.3 (Nguyen et al., 2015) with the optimized model and further visualized and annotated using EvolView (Zhang et al., 2012). Phylogenetic analysis based on ARG sequences used the maximum likelihood method. Bootstrap analysis was performed using 1000 replicates.

2.8. Accession number

The metagenomic and metatranscriptomic raw reads and assemblies analyzed in this paper were deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), with accession number PRJNA406858.

3. Results and discussion

3.1. Overview of metagenomic and metatranscriptomic analyses

This study utilized a deep metagenomic sequencing approach to

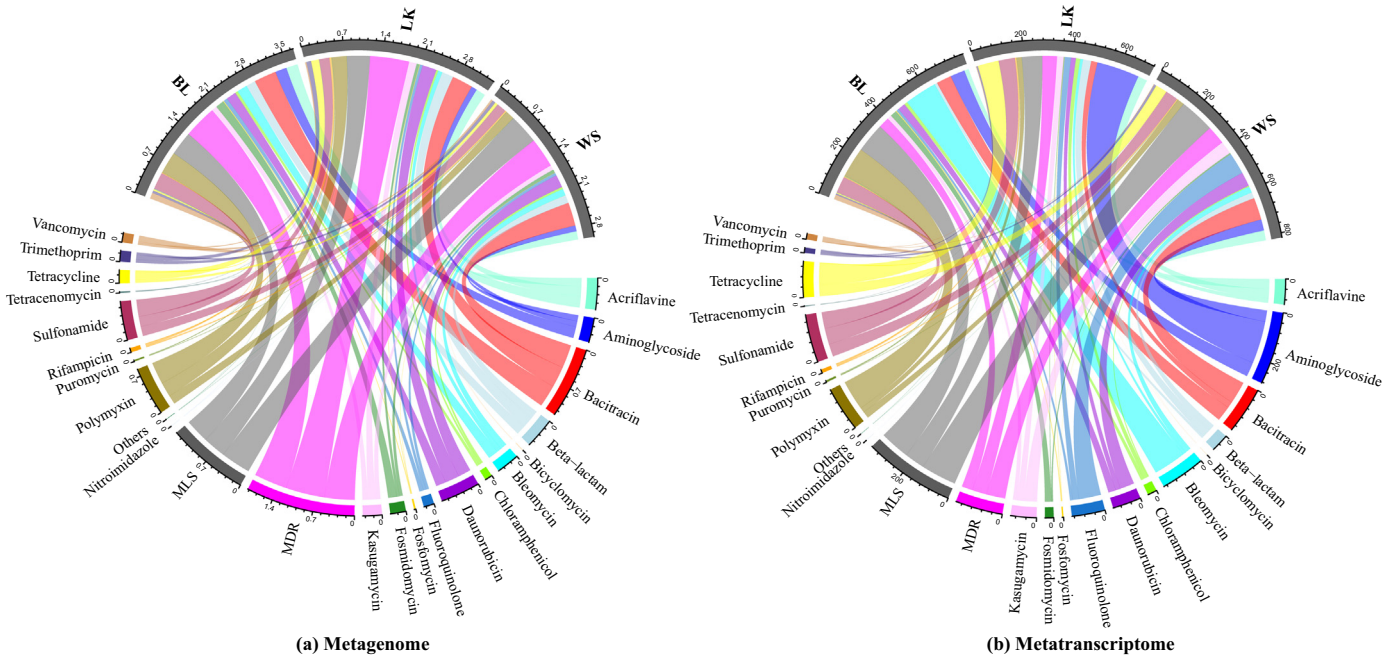

Fig. 1. Distribution of antibiotic classes in the three AS (a) metagenomes and (b) metatranscriptomes. The length of bars on the outer ring represents the abundance (ratio) and transcript abundance (×/Gb) of antibiotic classes in each AS metagenome and metatranscriptome. MLS: Macrolide-Lincosamide-Streptogramin; MDR: Multidrug.

examine the ARGs present in AS. Overall, Illumina sequencing generated > 77 Gb (giga base pairs) of raw reads for each AS sample, resulting in a total of 250 Gb (Table S3), which might be the largest metagenomic sequencing dataset for a single AS sample for ARG analysis to our knowledge. After assembly using IDBA-UD's assembly algorithm, about 941,290 (BL), 1,548,722 (LK) and 1,776,572 (WS) scaffolds longer than 500 bp were assembled from the metagenomic reads (Table S3). Further, a total of 1281 genomic bins were constructed from the metagenomes using a differential coverage binning approach. Similarly, unprecedentedly large metatranscriptomic data (> 58 Gb of raw reads for each AS sample) was employed to reveal the expression levels of identified ARGs. More information on the datasets, assembly and binning is provided in Supplementary Information.

3.2. Occurrence, abundance and diversity of ARGs

To identify the ARG sequences from metagenomic data, the ORFs of assembly scaffolds were aligned against the IARDB using BLASP. By using this method, 26 antibiotic classes' genes, consisting of 1237 ARGs (Table S2), can be analyzed simultaneously. Among the 26 antibiotic classes, 24 classes were detected in the three AS samples (Fig. 1a). Genes associated with aminoglycosides, acriflavines, bacitracin, beta-lactams, daunorubicin, multidrug resistance (MDR), macrolide-lincosamide-streptogramin (MLS), polymyxin and sulfonamide were more abundant and commonly distributed than other ARGs across the three AS samples. As expected, these abundant ARGs were usually associated with antibiotics used extensively in human or veterinary medicine including as growth promoters (Butaye et al., 2003; World Health Organization, 2017). Abundances of resistance to different antibiotic classes varied greatly, from a ratio of 7.06×10^{-1} for MDR to 1.20×10^{-4} for nitroimidazole (Table S4). In all samples, MDR was the most abundant class of resistance, with a ratio of 5.99×10^{-1}, 7.06×10^{-1}, and 5.41×10^{-1} in BL, LK, and WS, followed by MLS and bacitracin. While some previous reports confirm these 3 classes as highly abundant in AS of leachate treatment plants (Zhao et al., 2018), other studies showed aminoglycosides and tetracycline (Yang et al., 2013) or glycopeptides and beta-lactams (Guo et al., 2017) as the dominating classes of resistance. These reported variations in ARG composition across different AS samples might be associated with two main factors: (1) the different wastewater sources and treatment processes of WWTPs; (2) the completeness of ARG databases, depth of metagenomic sequencing and alignment similarity which influence the results of ARG analysis.

We further analyzed the composition of ARGs in the three AS samples. Of the 1237 target ARGs, a total of 360 were observed in these three AS, with an abundance range of 5.1×10^{-1}–3.20×10^{-5} ratio (Table S5). The community from LK, the municipal WWTP, displayed a slightly higher ARG diversity (284) than those from BL (201) and WS (234). This could be mainly because municipal WWTPs receive human fecal waste containing various types of antibiotic resistant bacteria and genes. The most abundant ARGs were similar across the three samples, still, a significant difference in ARG composition among these samples was detected ($p < 0.001$). Similarly, the analysis of high-throughput community sequencing data showed there was a distinct difference in microbial composition among the three different WWTPs sludge communities, especially at the species level ($p < 0.001$) (Supplementary Information). Different wastewater sources and treatment processes resulted in distinct differences of the bacterial community composition and further lead to the variation in ARG composition.

Eighty-one major ARGs reached an abundance of $> 1 \times 10^{-2}$ ratio in at least one sample (Fig. 2a). Among the top 20 ARGs, accounting for 68.6%–70.7% of the total ARG abundance, resistance to a multitude of antibiotic classes was found: acriflavine (*acrB*, *acrF*), bacitracin (*bacA*, *bceA*), beta-lactam (*pbp2*), bleomycin (*ble*), daunorubicin (*drrA*), fosmidomycin (*rosA*), kasugamycin (*ksgA*), MDR (*mdtB*, *mdtC*, *mexW*, *mexK*), MLS (*macA*, *macB*), polymyxin (*arnA*, *arnC*), sulfonamide (*sul1*, *sul2*), and trimethoprim (*dfrA3*) (Table S5). Concerningly, the gene *macB* conferring resistance to macrolides, one of the top 3 most prescribed antibiotic classes in human medicine (Public Health England, 2015), was extremely abundant across all three AS samples (3.29×10^{-1}–5.09×10^{-1}). Moreover, the resistance genes *bacA* and *sul2* associated with bacitracin and sulfonamide were also detected with remarkably high abundance. These three ARGs, especially the *macB* gene, are frequently detected with high abundance in various

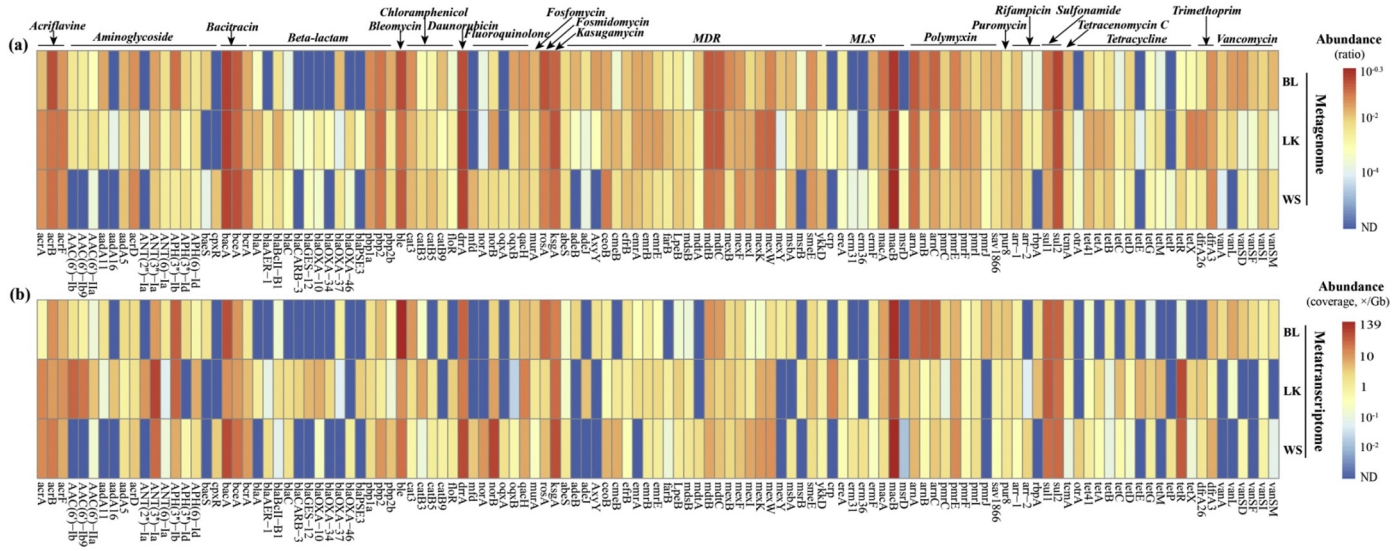

Fig. 2. Profile of 128 major ARGs in the three AS (a) metagenomes and (b) metatranscriptomes (abundance $> 1 \times 10^{-2}$ ratio or transcript abundance $> 1 \times 10^{-1}$ ×/Gb in at least one sample). ratio: copies of ARG per copy of 16S rRNA gene.

environments (Chen et al., 2013; dos Santos et al., 2017; Gao et al., 2018; Van Goethem et al., 2018; Zhang et al., 2015; Zhang et al., 2011), including AS (Guo et al., 2017; Li et al., 2015b; Zhao et al., 2018). However, the genes *macB* and *bacA* were observed with much higher abundance in this study's AS samples than reported previously, perhaps correlated to the large number of corresponding ARG reference sequences collected and added to the IARDB, and different alignment similarity used in this study. Furthermore, high diversity of tetracycline resistance genes was observed in our WWTPs. While not among the dominant ARGs detected, a total of 34 tetracycline resistance genes were detected, and among them 12 genes, for examples, *tet41*, *tetA*, *tetB*, *tetD*, *tetO* and *tetX*, were observed across all the samples, with an abundance range of 5.23×10^{-2}–9.85×10^{-5} ratio. The detection of various tetracycline resistance genes in our WWTPs may mainly be associated with the frequent use of tetracycline antibiotics for protection of human and animal health (Sarmah et al., 2006). Overall, by using of deep metagenomic sequencing and assembly, tremendously diverse ARGs were identified from different WWTPs AS, highlighting the fact that the AS is a hotspot of various ARGs, independent of wastewater source and treatment processes.

3.3. Occurrence and abundance of ARG transcripts

Since gene expression is in general a better proxy for functional activity than gene content (Sorek and Cossart, 2010), comprehensive metatranscriptome analysis can provide additional knowledge about resistance gene functioning and regulation. In our AS, all 24 identified classes of antibiotic resistance were observed in the metatranscriptomes with transcriptional activity (Fig. 1b). In general, genes conferring resistance to aminoglycosides, bacitracin, fluoroquinolone, polymyxin, MDR, MLS, sulfonamide, and tetracycline were detected with the highest transcript abundance (63.1%–76.4%) (Table S4). While most of these antibiotic classes were also dominant in the corresponding metagenomes, the expression ranks varied considerably in comparison to the abundance data (Fig. 1a & b). Still, at the antibiotic class level, there was significant ($p < 0.01$, Pearson correlation) correlation between observed and expressed resistance in BL and WS, while in LK this correlation is highly indicative, but not significant ($p = 0.06$).

At the ARG level, 237 of 360 (65.8%) identified ARGs showed transcriptional activity, with transcript abundance ranging from 1.39×10^{2} to 1.41×10^{-2} ×/Gb (Table S5). Overall, the resistance gene transcripts *macB*, *ble*, *bacA*, *drrA*, *ksgA*, *sul1*, *ANT(3″)-Ia*, *sul2*, *APH (3″)-Ib*, and *bceA* were the 10 most expressed ARGs across the three WWTPs AS (Fig. 2b). Notably, some highly transcribed ARGs were not predominant in the metagenomes. For instance, two aminoglycoside resistance genes *ANT(3″)-Ia* and *APH(3″)-Ib* were detected with a remarkably high transcript abundance across the three AS, despite their relatively low gene abundance (Fig. 2a). Moreover, *sul1* (34.48 ×/Gb) and *sul2* (28.29 ×/Gb) conferring resistance to sulfonamide were observed with similarly high transcript abundance, whereas the average abundance of *sul2* (1.79×10^{-1}) was far higher than *sul1* (3.71×10^{-2}) the metagenomes (Table S5). Further, *tetR* encoding a tetracycline repressor protein, which regulates transcription of tetracycline resistance determinants in Gram-negative bacteria (Berens and Hillen, 2003), displayed relatively high transcription, although its abundance was relatively low. Despite these few exceptions, transcript abundance of ARGs was overall still significantly ($p < 0.001$) positively correlated with the ARG abundance across all three WWTPs. Similar to our finding, a previous study, to our best knowledge the only published study so far, conducted correlation analysis between ARGs and their transcripts in water environments, and also demonstrated a significant correlation in both hospital and farm effluents ($p < 0.0001$) (Rowe et al., 2017). This indicated that the ARG abundance might be a dominant factor in affecting the expression levels of ARGs.

Despite a growing body of knowledge about distribution and abundance of ARGs in WWTPs, expression levels of the ARGs within WWTPs have barely been studied. In this study, the powerful combination of metagenomics and metatranscriptomics provides comprehensive insight into the expression of ARGs and we believe this to be the first report of the high level of the identified ARGs were being expressed in AS (65.8%). While our results indicated that the ARG abundance may be a dominant factor in affecting ARG transcription, it must be noted that there are many confounding factors (e.g. nutrients, heavy metals, antibiotics) that may be responsible for the observed overexpression of ARGs (Rowe et al., 2017), which may explain that some specific ARGs displayed high expression levels but relative low abundance in our AS samples. Furthermore, it is reasonable to correlate ARG transcription with metabolic activity of host. Therefore, some highly transcribed ARGs might be associated with nitrogen removal bacteria, as these bacteria were usually observed with high metabolic activity in WWTPs (Peng et al., 2015; Seuntjens et al., 2018; Yao and Peng, 2017). On the whole, metatranscriptomic analysis highlighted that ARGs were not only present, but also transcriptionally active in AS. In consideration that the active ARGs may have higher level of risk, it

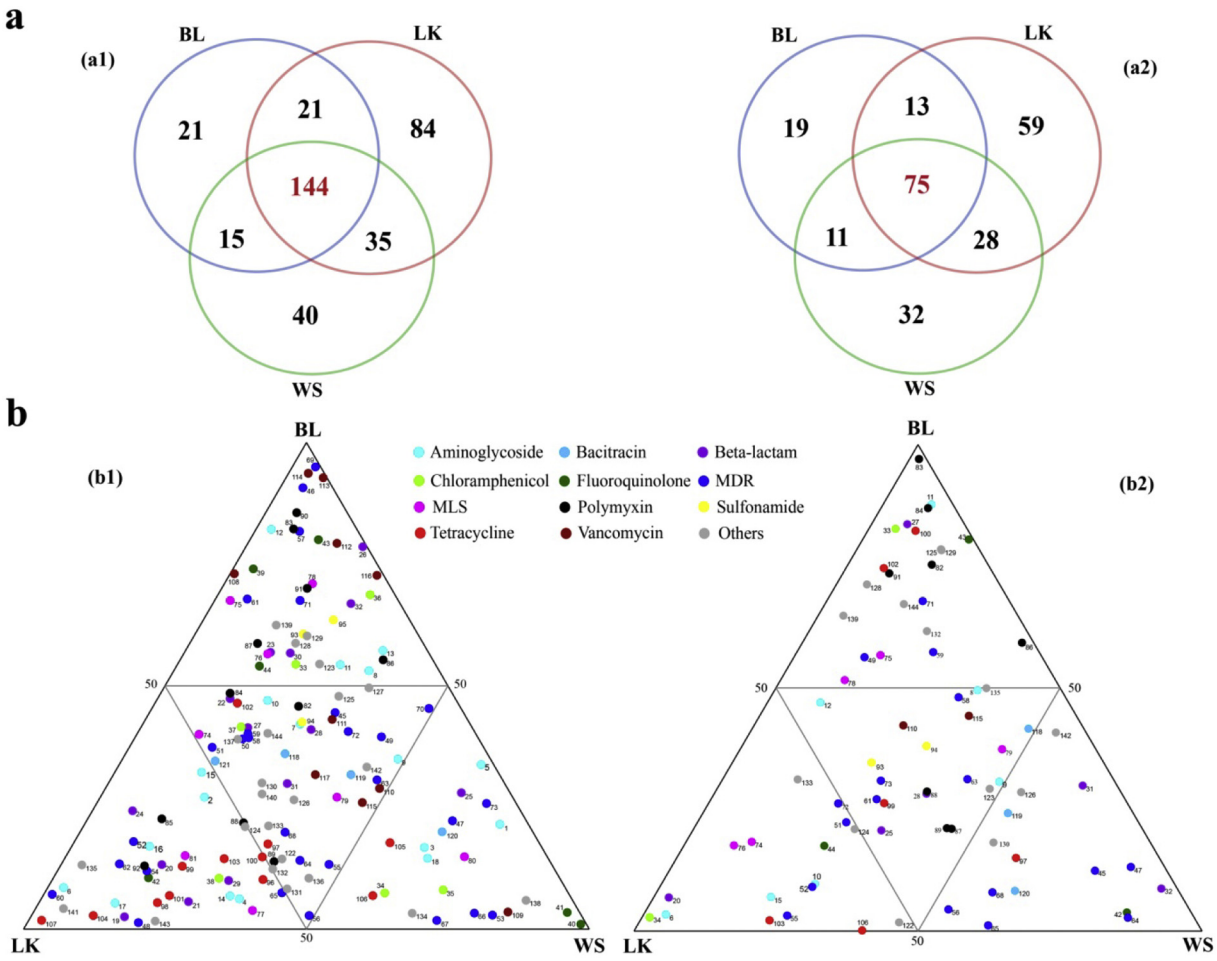

Fig. 3. Shared and unique ARGs and ARG transcripts. (a) Venn diagrams showing the number of shared and unique (a1) ARGs and (a2) ARG transcripts among the three AS samples. (b) Ternary plots showing the abundance comparison of shared (b1) ARGs and (b2) ARG transcripts. The codes in the ternary plot and the corresponding ARGs are listed in Table S6.

would be worthwhile to incorporate ARG expression data when doing the risk assessment of ARGs.

3.4. Shared ARGs and ARG transcripts among the three WWTPs

A vast majority of ARG and ARG transcripts were shared by all three samples from independent locations. 144 ARGs belonging to 20 antibiotic resistance classes were shared (Fig. 3a1), accounting for > 90% of total ARG abundance across the three samples. Among the 144 shared ARGs, the resistance genes of MDR, MLS, bacitracin, polymyxin, fosfomycin and sulfonamide were dominant across the AS samples. Fig. 3b1 further exhibited the detailed information on the shared ARGs and their abundance comparison among BL, LK and WS AS. At the ARG level, no significant difference ($p > 0.05$) in shared ARG composition among the three AS samples was observed, indicating that the core ARGs across AS samples, were relatively stable, despite obvious differences in influent wastewater and treatment processes.

Similarly, 75 shared ARG transcripts contributed to > 90% of the total ARG transcript abundance (Fig. 3a2). Again, no significant difference was found in shared ARG transcript compositions among the three samples ($p > 0.05$). From Fig. 3b1 and b2, we can find the abundance of genes and transcripts of most shared ARGs in each AS were consistent. Further, the correlation analysis indicated that the transcript abundance of shared ARGs was significantly ($p < 0.001$) positively correlated with their abundance across our AS samples. Based on the results presented above, we summarized that the shared ARGs contributed the overwhelming majority of total ARG abundance and transcripts in our AS.

3.5. Plasmid-associated ARGs

Plasmids play important roles in mobility and acquisition of ARGs and allow transfer to a wide variety of microorganisms via horizontal gene transfer (Klümper et al., 2017; Klümper et al., 2015; Musovic et al., 2014). In total, 128, 208, and 126 ACSs were annotated as plasmid-associated by comparison against the ACLAME database in BL, LK, and WS AS, respectively. Within these ACSs, 153, 252, and 143 ARG ORFs were annotated as plasmid-associated ARGs, accounting for 3.7–5.7%, of the total ARG ORFs in all three samples (Table 1). Overall, 110 different ARGs conferring resistance to 19 antibiotic classes were associated with plasmids (Fig. S4a). Genes conferring resistance to aminoglycoside, beta-lactam, MDR, and tetracycline were most commonly plasmid-associated, consistent with previous studies

Table 1
ARG ORFs encoded by plasmids and chromosomally.

WWTPs	Plasmid-associated ARG ORFs		Chromosomally-associated ARG ORFs	
	ARG	ARG transcript	ARG	ARG transcript
BL	153 (5.7%)	60 (6.9%)	2510 (94.3%)	814 (93.1%)
LK	252 (5.2%)	124 (10.7%)	4557 (94.8%)	1040 (89.3%)
WS	143 (3.7%)	80 (5.8%)	3769 (96.3%)	1290 (94.2%)

(Kristiansson et al., 2011; Szczepanowski et al., 2009; Zhang et al., 2011). Furthermore, among the ARG-carrying plasmid-associated sequences, 12 scaffolds were observed to carry 3 or more ARGs. This strongly indicates the presence of multidrug-resistance plasmids in AS, especially considering that each scaffold is only representing a partial sequence of a plasmid potentially hosting further resistance genes. For example, scaffold LK_47911 with a length of 5808 bp, was found to carry aminoglycoside resistance gene (*ANT(3″)-Ia*), beta-lactam resistance genes (*blaA* and *blaOXA-34*), and MLS resistance genes (*ereA* and *lnuF*). By comparison against the NCBI nucleotide database, this scaffold shares high homology with the complete sequence of the plasmid pSE15-SA01028 (GenBank accession number NZ_CP026661.1) carried by *Salmonella enterica*, a common human pathogen (Vågene et al., 2018).

Unlike previous studies conducted to explore the presence of ARGs in plasmids using culture-dependent or metagenomic methods, our study expands further effort to illustrate not only abundance but also expression of these ARGs. Among the 110 plasmid-associated ARGs, 77 were detected with transcriptional activity (Fig. S4). Specifically, 264 of 548 plasmid-associated ARG ORFs were observed to have transcriptional activity, accounting for 48.2% of the total ARG ORFs encoded by plasmids, whereas only 29% (3144 of 10,836) of the chromosomally encoded ARG ORFs were being expressed (Table 1). Thus, the likelihood of ARGs to be expressed close to doubles when encoded by a plasmid (Fig. S5). More specifically, the previously mentioned high transcript abundance of aminoglycoside, sulfonamide, and tetracycline resistance genes was mainly associated with plasmid-borne ARGs (Fig. 4).

Previous studies mainly emphasized the role of plasmids in horizontal transfer of various ARGs (Bennett, 2008; Kristiansson et al., 2011; Zhang et al., 2011), information regarding the expression of plasmid encoded ARGs is scarce. With this in mind, we investigated the expression levels of plasmid-encoded ARGs based on metatranscriptomic data. Interestingly, we found that compared to chromosomally encoded ARGs, the ARGs within plasmids were observed to display a far higher transcription probability and contributed more notable transcript abundance, which has never been reported previously. This novel finding implied that ARG-carrying plasmid may play a much more important role in conferring resistance to antibiotics and is at greater risk than has been realized. The high transcript level of plasmid-borne ARGs, such as aminoglycoside, sulfonamide, or tetracycline resistance genes, may be either associated with the presence of corresponding antibiotics or more likely with the presence of more strongly transcribed constitutive promoters being present on plasmids. Overall, the ARGs encoded by plasmids deserve special attention not only due to their transferability, but also their high level of expression.

3.6. Identification of ARG hosts by binning analysis

Previous studies mainly used network analysis to explore the distribution of ARGs across potential hosts in metagenomic data (Jia et al., 2017; Li et al., 2015b; Su et al., 2017), however, such method can only reveal limited ARGs hosts and need to be further validated (Li et al., 2015b; Zhao et al., 2018). Recently, several studies investigated ORFs of ARGs in assembled contigs from metagenomic data (Chu et al., 2018; Jia et al., 2017; Ma et al., 2015; Van Goethem et al., 2018). Nevertheless, owing to the relative low sequencing depth, only a small number of ACSs were detected and assigned to hosts. In this study, based on deep sequencing data and binning method, 635 high-quality ARG carrying genome (ACG) bins carrying 159 different ARGs were identified from the whole metagenomes (Table S7). Among them, 324 bins (51%) could be annotated at the genus, 468 (73.7%) at the family, and 620 (97.6%) at the phylum level (Table S8). In total, > 22 bacterial phyla were detected as the hosts of the bin-associated ARGs, with

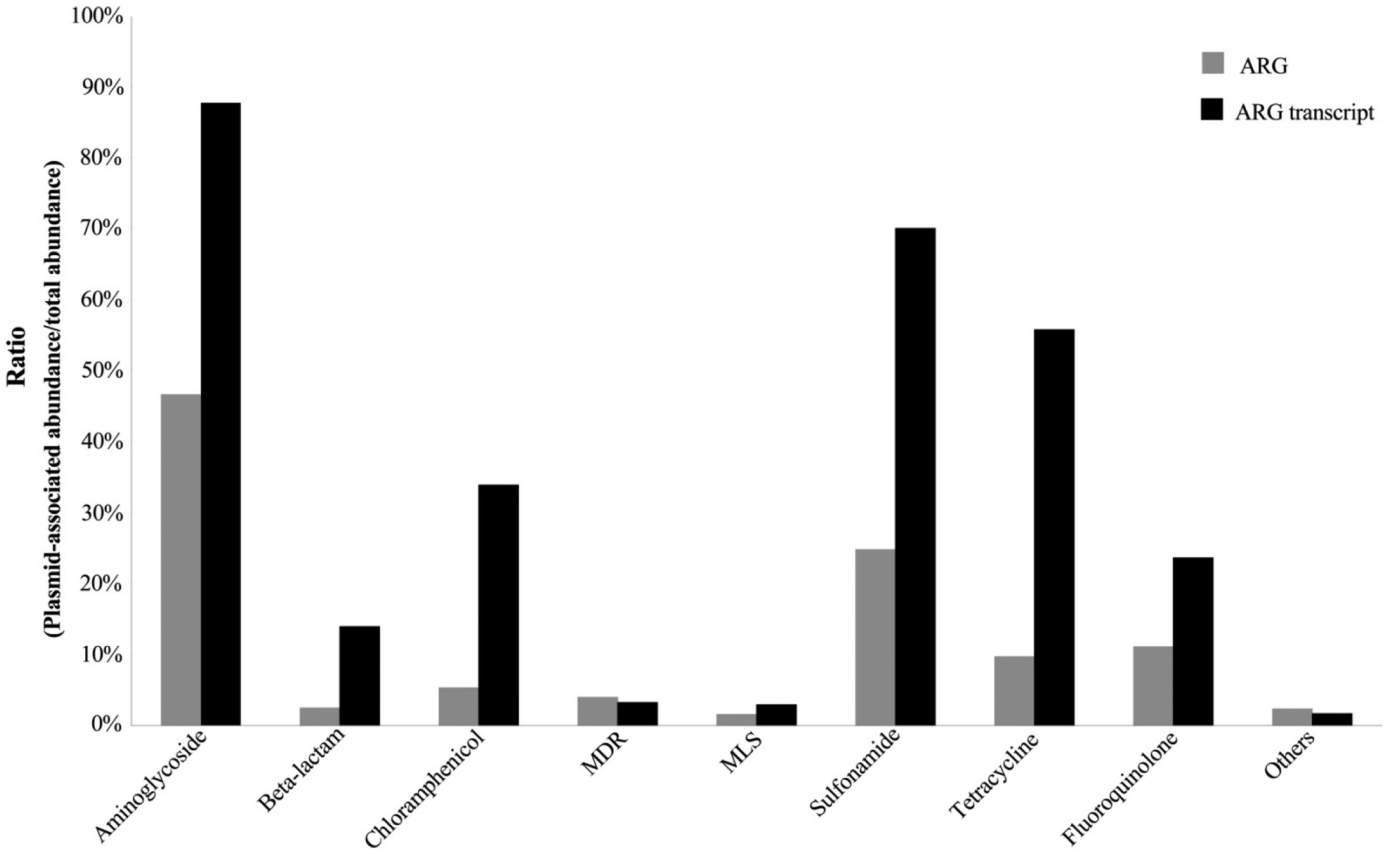

Fig. 4. The average contributions of plasmid-associated ARG to each antibiotic type abundance and transcript abundance.

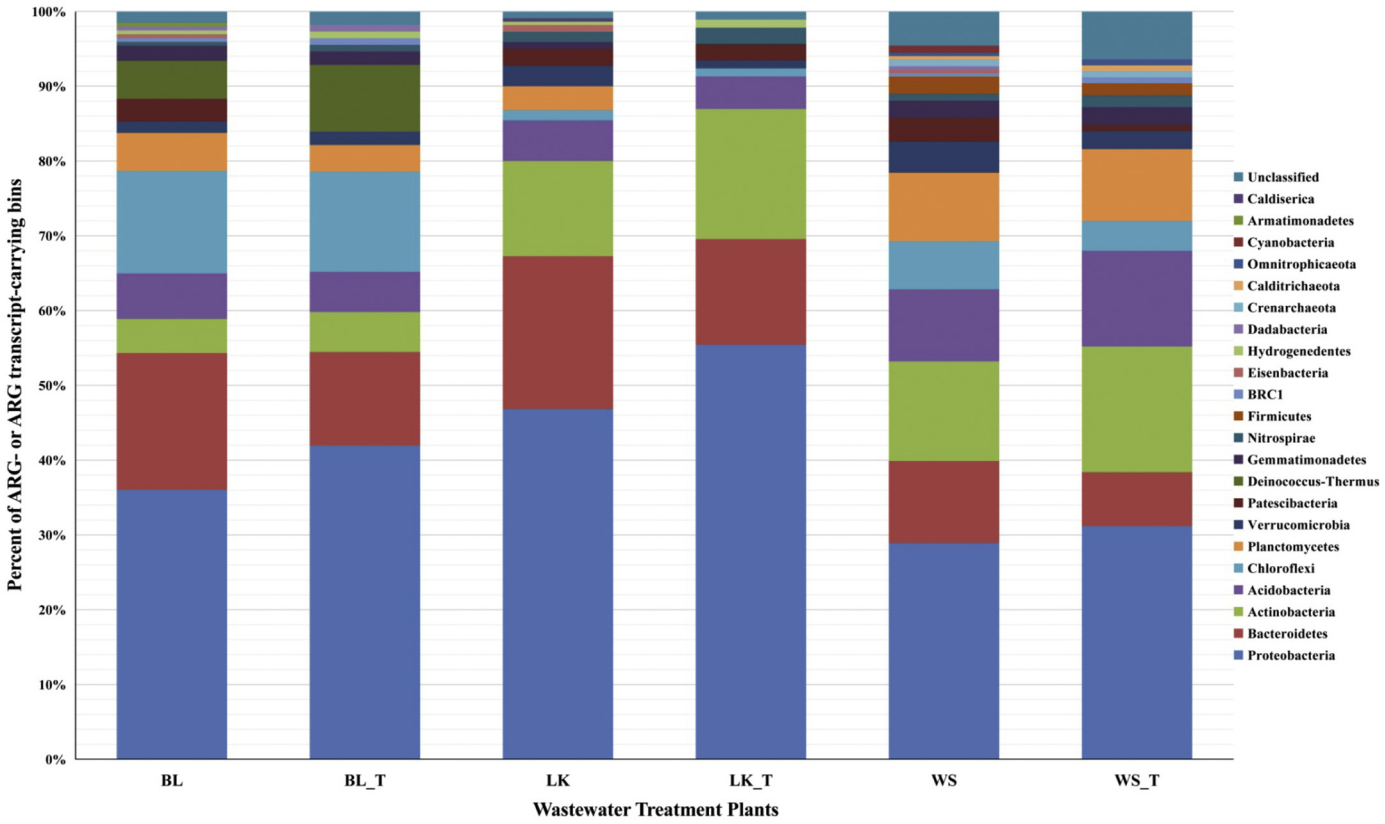

Fig. 5. Phylum level composition of ARG- and ARG transcript-carrying genome bins reconstructed from the AS metagenomes. BL_T, LK_T and WS_T showed the phylum composition of ARG transcript-carrying genome bins from BL, LK and WS, respectively.

Proteobacteria being observed most frequently, followed by *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Planctomycetes* (Fig. 5). Linked with microbial community composition, unsurprisingly, the major phyla in ARB were established as the dominant phyla in our AS bacterial community (Supplementary information, Figs. 5 and S6). The abundance of ARGs carried by these dominant phyla occupied the vast majority of the total bin-associated ARGs. For example, Proteobacteria-related ARGs accounted for 40.1%, 70.8%, and 44.4% of the total bin-associated ARG abundance in BL, LK, and WS, respectively (Fig. S7). Pearson correlation analysis showed the abundance of the ARGs was significantly positively correlated ($p < 0.01$) with their host phyla abundance.

It should also be pointed out that *Firmicutes*, one of the most abundant phyla in all three AS communities, contributed to a disproportionately low ARG abundance (Figs. S6 & 7), despite some genera within this phylum, such as *Ureibacillus* and *Bacillus*, being frequent associated with antibiotic resistance in other studies (Perreten et al., 2005; Wang et al., 2015). Out of the 635 total bins, 329 displayed active ARG transcription (Table S9). The taxonomic composition of ARG transcript-carrying genome bins was remarkably consistent with ACG bins in each AS sample (Fig. 5). With a few exceptions, the most abundant phyla again contributed the most ARG transcripts (Fig. S7). For instance, the phylum *Nitrospirae* contributed relatively high numbers in ARG transcript abundance but low ARG abundance in the LK sample (Fig. S7b). This might be directly linked to the anaerobic nature of this sample, where species belonging to *Nitrospirae* usually show high levels of nitrogen removal activity in WWTP (Peng et al., 2015; Seuntjens et al., 2018; Yao and Peng, 2017). Differential ARG transcription can thus be directly linked to certain processes of the wastewater treatment process.

At the family level, 128 families of bacteria belonging to 19 phyla were explored to be the hosts of 146 ARGs (Table S10). Among them, *Burkholderiaceae*, *Rhodocyclaceae*, *Sphingomonadaceae*, and *Steroidobacteraceae*, families reported to be the important hosts of ARGs previously (Liu and Pop, 2008; Selvaraj et al., 2018; Singh et al., 2008), harbored > 20 ARGs (Table S10). Similarly, these bacterial families also harbored most diverse expressed ARGs (Table S11). Most notably, the family *Burkholderiaceae*, which contains truly environmental saprophytic organisms, phytopathogens, opportunistic pathogens, as well as primary pathogens for humans and animals, was detected as the host of 50 ARGs. Moreover, 32 of the 50 ARGs in *Burkholderiaceae* were detected with transcriptional activity (Table S11). These findings suggested that *Burkholderiaceae* are the most important host of ARGs in our WWTPs AS and transmission of antibiotic resistant *Burkholderiaceae* from WWTPs to the environment might pose a suitable target for risk assessment studies.

Owing to the low completeness of ARG host's draft genome or limitation of reference sequences for taxonomy annotation, only 51% of hosts can be annotated at genus level (Table S8). This limitation is common in taxonomic identification of genomes generated from metagenomic data (Ma et al., 2015). Despite this, this study still revealed that 165 bacteria genera were the hosts of 112 ARG types (Table S8). A considerable fraction of these bacterial genera have not been reported as the ARG hosts, thus, this study greatly expands the knowledge of the diversity of ARB in WWTPs.

3.7. Multi-drug resistant host

Multi-drug resistant bacteria have become a major public health concern in many countries as there are fewer effective antimicrobial agents to treat infections caused by these bacteria (Kumarasamy et al., 2010; Pitout and Laupland, 2008). Among the 635 ACG bins, more than half carried two or more ARGs. 107 bins which carried four or more ARGs were considered representative multi-drug resistant ACG bins

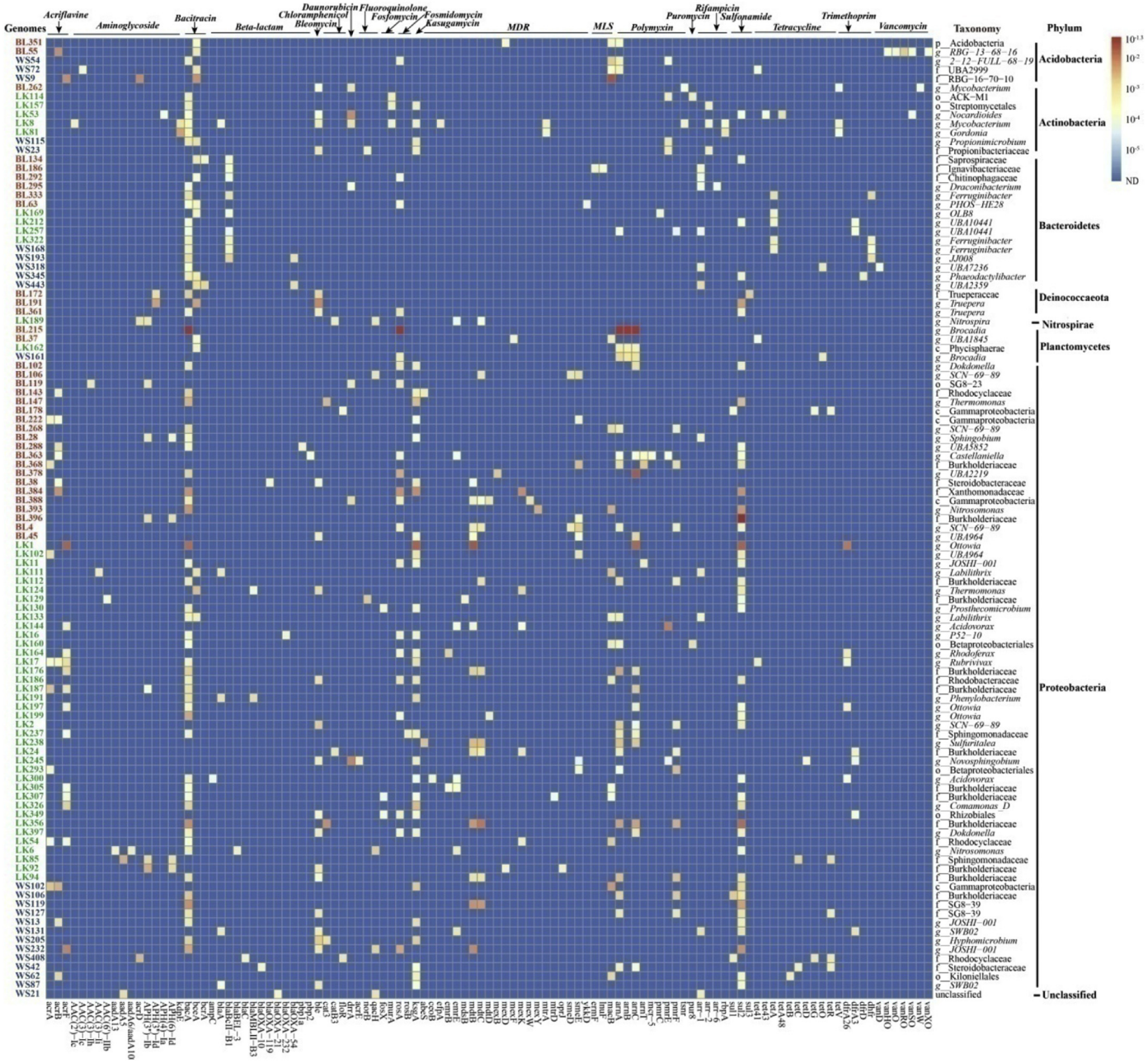

Fig. 6. Distribution and abundance of ARGs in the representative ACG bins (carrying ≥ 4 ARGs). Red, green and blue indicate genome bins reconstructed from BL, LK, and WS. p: phylum; c: class; o: order; f: family; g: genus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 6). Seventy of these bins (65.4%) were annotated to *Proteobacteria*. Further, 14, 8, and 5 representative bins were annotated to *Bacteroidetes*, *Actinobacteria*, and *Acidobacteria*, respectively (Fig. 6). Overall, the multiple ACG bins belonging to these four phyla occupied > 90% of the representative bins. Furthermore, it is worth noting that a genome bin LK8 from municipal WWTP (LK) and annotated as genus *Mycobacterium*, harboring 14 ARGs, which conferred resistance to a total of 10 different antibiotics. Further analysis showed 6 of these 14 ARGs could be detected with transcriptional activity (Fig. S8). The actinobacterial genus *Mycobacterium* includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) in humans (Rahul and Nishy, 2016). Therefore, *Mycobacterium*, most possibly from municipal wastewater, harboring high numbers of ARGs in WWTPs might deserve future attention.

Surprisingly, 4 representative ACG bins were annotated as *Planctomycetes*, with 2 of them annotated as genus *Brocadia*. Strikingly, 5 ARGs carried by genus *Brocadia* (BL215) were detected with extremely high relative abundance (Fig. 6), as well as transcript abundance (Fig. S8). This can be well explained by the fact that *Brocadia*, the host of these 5 ARGs, is one of the top 3 abundant genera in the BL sample. As a genus carrying out anaerobic ammonium oxidation (Anammox) activity, *Brocadia* is widely distributed in WWTPs and plays an important role in nitrogen removal from waste streams (Kartal et al., 2010). Here, we revealed for the first time that *Brocadia* could further serve as an important ARG host in WWTPs.

3.8. Correlation of ARGs with microbial phylogeny

Microbial community composition may be the dominant factors in

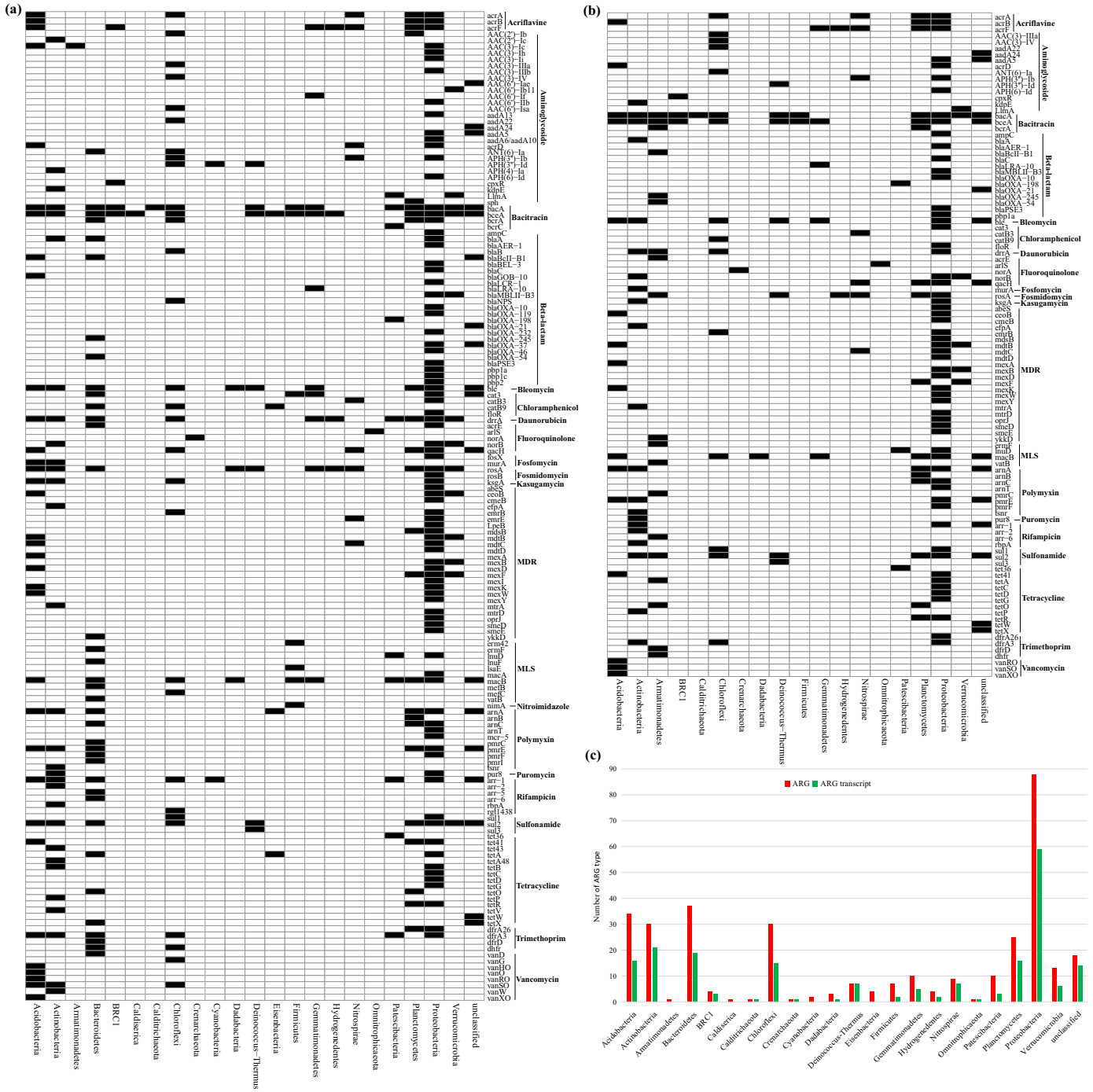

Fig. 7. Distribution of (a) ARGs and (b) ARG transcripts in each bacterial phylum. (c) Number of ARG and ARG transcript types in each bacterial phylum.

ARG distribution in wastewater environments, a speculation recently given by procrustes analysis (Zhao et al., 2018). Consistent with this speculation, Chen et al. (2017) suggested that ARG profiles were significantly ($p < 0.001$) correlated with bacterial community composition in groundwater near a municipal solid waste landfill, and hypothesized that bacterial community shift is the major driver shaping the ARG distribution. In addition, two recent studies also indicated that the sewage treatment plant core resistome is linked to the microbial community significantly ($p < 0.001$) (Munck et al., 2015; Su et al., 2017). Nevertheless these results were all based on mathematical statistics, their robustness and reliability in predicting the correlation between ARG distribution and microbial community need to be further confirmed. In this study, by means of metagenomic assembly and binning approaches, it is possible for us to reveal the distribution of ARGs in bacterial taxa. Fig. 5a exhibited the distribution of 159 ARGs in 22 bacterial phyla. The phylum *Proteobacteria* harbored most diverse ARGs (88 ARGs), followed by *Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, and *Actinobacteria*, carrying 37, 34, 30, and 30 ARGs, respectively (Fig. 7c). However, only 7 ARGs were shared by these 5 primary ARG-carrying phyla, and with each phylum carrying a significant number of unique ARGs (Fig. S9). In total, 99 ARGs (62.3%) were carried by unique host phyla. These results implied that microbial taxa are mainly associated with their individual ARGs, which is highly consistent with the theory that some specific microbial taxa carry specific ARGs (Forsberg et al., 2014). Therefore, our results presented here provided strong evidence for the hypothesis that microbial phylogeny drives the

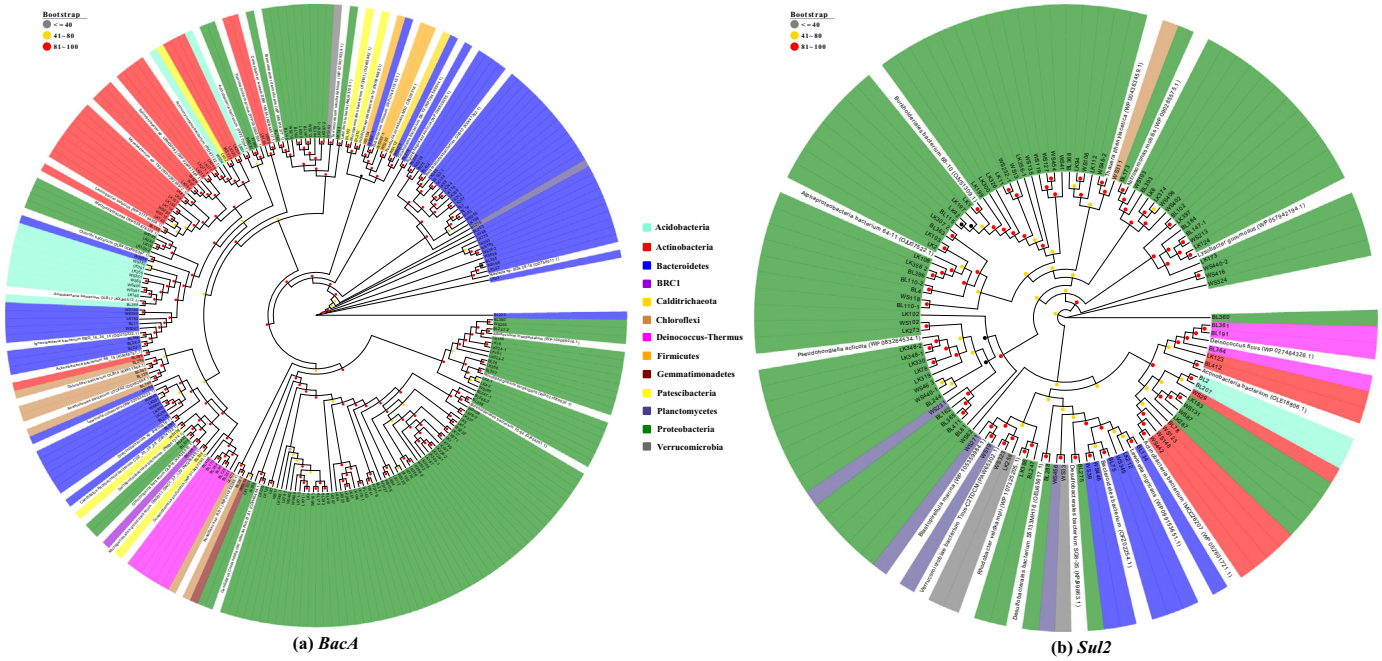

Fig. 8. Phylogenetic analysis of ARG sequences identified from draft genomes across the three AS metagenomes. (a) Bacitracin resistance gene (*bacA*); (b) Sulfonamide resistance gene (*sul2*). Reference sequences are provided with accession numbers. ARG sequence(s) from same bacterial phyla were highlighted with same background color.

distribution of ARGs in WWTPs.

Of the 159 ARGs assigned to microbial taxa, the resistance genes *bceA*, *bacA*, *rosA*, *drrA*, *ble*, *macB*, and *sul2* were carried by the most diverse hosts (Fig. 7a). Similarly, transcripts of these ARGs were observed most frequently associated with different hosts (Fig. 7b). For the ARGs carried by a broad range of hosts, we speculate that some of them may be intrinsic in bacteria. However, more data is required to validate this speculation. Of particular concern is that the bacitracin resistance gene *bacA* and sulfonamide resistance gene *sul2*, which have been reported in various environmental samples and often detected with a very high relative abundance (Gao et al., 2018; Li et al., 2015b; Wei et al., 2018; Zhao et al., 2018), were carried by 14 and 9 bacterial phyla and further detected with transcriptional activity in 11 and 7 phyla respectively in our AS samples (Fig. 7a & b). To test the hypothesis that phylogeny, rather than horizontal gene transfer (HGT), drives the diversity of these two ARGs, we next evaluated the evolutionary history of these genes by constructing phylogenetic trees consisting of *bacA* or *sul2* ORFs in ACG bins and reference ARG protein sequences from known bacteria (Fig. 8). Phylogenetic analyses showed both ARGs displayed high protein diversity, even within the same phyla. However, ARG sequences derived from the same or closely related host taxa clustered distinctly. These results indicated that both the resistance genes *bacA* and *sul2* might be conserved in host species at low taxonomic level, though *sul2* was frequently associated with mobile genetic elements (Kristiansson et al., 2011; Zhang et al., 2018; Zhang et al., 2011), which implied microbial phylogeny is the major factor that affected the diversity of these two ARGs in our AS. Additionally, many *bacA* or *sul2* sequences shared high homology across different AS samples, implying that the spread of these two ARGs may occur in immediate linkage to their hosts.

4. Conclusion

Based on the large metagenomic datasets and binning method, we present the distribution of ARGs in bacterial draft genomes in AS, thus far extending the resolution achieved using traditional PCR/qPCR approaches or short read-based metagenomic analysis. By combining metagenomic and metatranscriptomic analyses, our results indicated that 65.8% of identified ARGs were not only present, but also actively transcribed, and their transcript abundance positively correlated with ARG abundance significantly ($p < 0.001$). Most interestingly, plasmid-associated ARGs were observed to display a far higher transcription probability than ARGs encoded by chromosomes. Further, we demonstrated that ARGs were present and expressed in a broad range of bacterial phyla in AS, and 22 phyla, 128 families and 165 genera of bacteria were identified as the putative ARG hosts. Among them, genus *Mycobacterium* and family *Burkholderiaceae* were identified as the most important multi-drug resistant bacteria, harboring 14 and 50 ARGs respectively. This study increases our understanding of the WWTP as a hotspot of ARGs, further studies are required to better understand the fates of highly-expressed ARGs and multi-drug resistant hosts from WWTP to the environment.

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Appendix A. Supplementary data

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