Environmental Research 213 (2022) 113741

Contents lists available at ScienceDirect

Environmental Research

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

Metagenomics and metatranscriptomics analyses of antibiotic synthesis in activated sludge

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A R T I C L E I N F O

Keywords:
Antibiotic biosynthetic genes
Antibiotic resistance genes
Biofilm
Wastewater treatment plants

A B S T R A C T

The generic of antibiotics is considered to be a main reason for the generation of antibiotic resistance genes (ARGs) in wastewater treatment plants (WWTPs). However, little has been reported about the antibiotic biosynthesis by activated sludge. In this study, the distribution and expression of antibiotic biosynthetic genes (ABGs) in the floc sludge and biofilm from two WWTPs were deciphered using metagenomics and metatranscriptomics. The results showed that 2% of the community were in general well-linked to antibiotic production, indicating a non-negligible antibiotic synthetic ability of WWTPs. 93 ABGs belonging to 26 antibiotics were determined, among which aminoglycosides, β-lactams, ansamycins, peptides, macrolides were majority. The relative abundances of detected ABGs had a large interval, ranging from 0.000006% to 0.042%. The predominant antibiotic types of synthetic genes with higher relative expression levels were monobactams, penicillin & cephalosporins and streptomycin, primarily belonging to β-lactams and aminoglycosides. The hypothetical synthetic pathways of streptomycin synthesis and penicillin & cephalosporin synthesis were proposed. And the coexistence of ABGs and ARGs for these two antibiotics was also pronounced in activated sludge from meta-omics data. These findings for the first time demonstrated the antibiotic synthetic potential in activated sludges, revealing new sources of antibiotics and resistance genes in WWTPs, and thereby aggravating environmental pollution.

1. Introduction

Antibiotics are widely used in the prevention and treatment of biological diseases (Liu et al., 2022). Antibiotic resistance has become a serious threat to global public health. Antibiotic resistance genes (ARGs) spread as an emerging environmental pollutant in different environmental media, which may cause greater environmental implication than antibiotics themselves. ARGs are the culprits for antibiotic resistance of microorganisms, especially if they are present in pathogenic bacteria, which can create serious environmental health risks. Municipal wastewater treatment plants (WWTPs) collected a large amount of domestic and industrial wastewater and facilitate the proliferation and transfer of ARGs due to the rich nutrients and close interaction of microorganisms in WWTPs (Osińska et al., 2020). This unique environment containing multiple compounds may pose a serious threat to the spread of resistance (Karkman et al., 2018). Therefore, WWTPs are recognized as one of the most important routes to propagate ARGs, while wastewater and sludges are considered as the main pollution sources of ARGs in the environment (Pärnänen et al., 2019).

Typically, conventional WWTPs are effective in removing phosphorus, nitrogen and chemical oxygen demand (COD) from wastewater (Pronk et al., 2015), but antibiotics have a removal efficiency of 79%–88% (Pärnänen et al., 2019). Antibiotic occurrence in the WWTPs promotes the production and propagation of ARGs in activated sludge (Bengtsson-Palme et al., 2019; Zhao et al., 2019). Previous studies have shown that the concentration of some antibiotics (e.g., erythromycin, cloxacillin, penicillin) in the effluent of secondary treatment is still high, even higher than those in the influent. That is, removal rate below 0% (Gulkowska et al., 2008; Zhang and Li, 2011; Yan et al., 2014; Zhang et al., 2015; Kortesmaki et al., 2020; Bai et al., 2021). One reason is that the conventional biological treatment processes cannot completely remove antibiotics (Oberoi et al., 2019). Another important factor may be the existence of antibiotic biosynthesis in activated sludge. Antibiotic synthesizing microorganisms are widely distributed in water and soil environments (Bao et al., 2021). For example, *Actinomyces*, as major antibiotic producers, not only have extremely high biosynthetic potential in soil, but also have non-negligible abundance levels in activated sludge and may produce certain levels of antibiotics around them (Ike

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https://doi.org/10.1016/j.envres.2022.113741
Received 10 April 2022; Received in revised form 23 May 2022; Accepted 18 June 2022
Available online 22 June 2022
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et al., 2010; Liu et al., 2017). Low-level antibiotic can induce other bacteria to evolve high-level resistance (Wistrand-Yuen et al., 2018). Moreover, ARGs can also be disseminated from antibiotic producers to pathogens via mobile genetic elements (Jiang et al., 2017). Thus, antibiotic synthetic microorganisms may also play an important role in the spread of ARGs in WWTPs. It is difficult to determine the content of antibiotics produced by activated sludge due to the interference of influent antibiotics. Therefore, investigation of the abundance and expression of antibiotic biosynthesis genes (ABGs) in activated sludge is necessary to evaluate the potential of antibiotic biosynthesis of WWTPs.

The aim of the study was to investigate the antibiotic synthetic capacity of activated sludge. Biofilm and floc sludge samples from two WWTPs were analyzed using metagenomic sequencing for confirming the relative abundance of different types of ABGs and ARGs. Metatranscriptomic approaches were used to confirm the activity of the detected ARGs and ABGs, to further reveal the mechanisms of synthesis of several major antibiotics in activated sludge and to estimate the potential contribution of antibiotic producers to antibiotic synthesis.

2. Materials and methods

2.1. Sample collection

In this study, samples were collected from WWTP-A in Xiangtan City and WWTP-B in Zhuzhou City, Hunan Province, China. With a flux capacity of 100,000 m^3/day, the major biological processes involved in two WWTPs are those of anaerobic-anoxic-oxic integrating aerobic moving bed biofilm reactor (AAO-MBBR) and AAO integrating membrane bioreactor (AAO-MBR). Detailed information about these two WWTPs were shown in Table S1. Triplicate samples of floc sludge and biofilm were collected from the aeration tanks of WWTP-A (A-Sludge and A-Biofilm) and WWTP-B (B-Sludge and B-Biofilm) at different locations. There were 12 samples for metagenomic sequencing. Six of the floc sludge samples from WWTP-A and WWTP-B were used for metatranscriptomics sequencing. Finally, the collected samples were snap frozen in liquid nitrogen, stored in containers at −80 °C before further analysis.

2.2. DNA extraction and metagenomic sequencing

The metagenomics analysis was performed by Sangon Biotech (Shanghai) Co., Ltd. The standard procedures of OMEGA's E.Z.N.A.® Soil DNA Kit were used to extract DNA. In this experiment, extraction was performed using the magnetic bead method. The microbial cells are decomposed and digested using lysis solution and protease. The binding buffer and magnetic beads were used to adsorb pure DNA. After washing and elution, the DNA quality was verified by 1% agarose gel electrophoresis. Microbial genomic DNA was taken from each sample to build a metagenomic library.

A library of qualified genomic DNA in the samples was constructed. Use a new generation of library building kits and high-quality enzymatic components, improved adaptor connection technology, and high-fidelity enzymes with strong amplification efficiency for library enrichment. PCR-free amplification extended sequence coverage for simple, easy and efficient preparation of DNA libraries for the Illumina HiSeq platform (https://www.illumina.com/). The metagenomic sequencing flow is shown in Fig. S1(a). The origin sequence data have been deposited in the Genome Sequence Archive (accession number: CRA003823, https://bigd.big.ac.cn/).

2.3. RNA extraction and metatranscriptomic sequencing analysis

The scraping process of biofilm was time-consuming, and it was verified by quality inspection that total RNA degradation was obvious. Therefore, only floc sludge of WWTP-A and WWTP-B were taken in this study to determine the expression of ABGs and ARGs. RNA extracted from the samples was detected using 1% agarose gel electrophoresis. Useless rRNA (ribosomal RNA) was removed from the samples by using Ribo-Zero rRNA Removal Kits reagent. Addition of TruSeq™ RNA Sample Prep Kit and random primers to interrupt the target RNA and make the random primers complementary to the target RNA. PCR amplification was used to construct the transcriptome library. Denaturation with sodium hydroxide produces single-stranded DNA fragments. Finally, DNA clusters are generated by "bridge" PCR amplification. The metatranscriptomics sequencing flow is shown in Fig. S1(b).The raw RNA sequences data were deposited in the Genome Sequence Archive (https://bigd.big.ac.cn/, accession number: CRA007158).

2.4. Bioinformatic analysis

DIAMOND software (http://www.crystalimpact.com/diamond) was used to compare the protein sequences of gene set with the ARDB database to obtain the type and number of the corresponding ARG, functional annotation and homologous species information. Filter conditions were: E-value < 1e-5, Score >60. And the clean reads of the four types of samples were compared with the non-redundant gene set by using Bowtie2 (http://bowtie-bio.sourceforge.net/). The gene abundance of each sample was calculated by SAMtools (http://www.htslib.org/) according to the length of the gene and blasting read obtained. Functional annotation information of gene set was obtained via blasting the NCBI, KEGG (Kyoto Encyclopedia of Genes and Genome), SEED databases and ARDB (Antibiotic Resistance Genes Database). Function gene abundance and species abundance were obtained according to gene set abundance.

Similarly, the transcriptome was used to match the gene set with the nr, KEGG and ARDB using BLASTP (BLAST matching parameters were set with an expectation e-value of 1e-5, the same below) to annotate the genes with species and function.

ORF (open reading frame) prediction of contigs from the splicing results was performed using MetaGeneMark (http://exon.gatech.edu/meta_gmhmmp.cgi). Genes with nucleic acid length of more than or equal to 100 bp were selected and translated into amino acid sequences. The predicted gene sequences of all samples were clustered (parameters: 95% identity, 90% coverage) using CD-HIT (http://www.bioinformatics.org/cd-hit/), and the longest gene of each class was taken as the representative sequence to construct a non-redundant gene set. The aim of the metagenome-centric metatranscriptomics analysis was to identify highly expressed gene sets, as this provides an indicator of antibiotic synthesis in activated sludge chases. In RNA-seq analysis, the expression levels of genes were calculated by comparing the number of sequences (clean reads) to a reference genomic region. The transcripts per million (TPM) value of each gene/transcript in the sample was calculated using kallisto (https://pachterlab.github.io/kallisto/) and this value was taken as the expression of the gene/transcript in the sample.

2.5. Network analysis

The co-occurrence patterns between ARG and ABG were investigated by calculating all paired Spearman rank correlations among ARG subtypes and ABG subtypes for the same type of antibiotics. Performance of network analysis in R environment using vegan, igraph package and then visualized on Gephi 0.9.1 (https://gephi.org/). The statistical examination and calculation of the observed (O%) and random incidences (R%) of co-occurrence correlation between ARG and ABG were performed in the network. The degree of discrepancy between O% and R% (O/R ratio) was the reference baseline for examining non-random co-occurrence patterns (Ju et al., 2016).

3. Results

3.1. The profiling of known antibiotic producers in WWTPs

Taxonomic profiling of metagenomic and metatranscriptomic data based on the NCBI-nr database showed the known antibiotic producers including bacteria and fungi (Fig. 1), and the relative abundance of bacteria was much more than fungi. As shown in Fig. 1a, the total relative abundance of the known antibiotic-producing microorganisms in WWTPs was close to 2%, suggesting a non-negligible potential for antibiotic synthesis. The total relative abundance of these microorganisms in WWTP-B was higher than that of WWTP-A. The community composition of A-Biofilm and A-Sludge was different, whereas that of B-Biofilm and B-Sludge was very similar, indicating that the biofilm in WWTP-B was likely to originate from biocake. The results showed that *Pseudomonas*, *Burkholderia*, *Bacillus* and *Streptomyces* were the dominated antibiotic-producing bacteria in WWTPs, and their relative abundances were significantly different between the two WWTPs ($P <$ 0.05). *Pseudomonas* can produce a variety of antibiotics, like phenazines and pyoluteorin. 12 actinomycetes were found to be present in activated sludge, and *Streptomyces* were predominant actinomycetes in all samples and can synthesize a wide range of antibiotics, such as streptomycin, tetracycline, vancomycin, etc. *Burkholderia* had the ability to synthesize multiple antimicrobial compounds to fungi and bacteria, such as pyrrolnitrin, occidiofungins and betulinans (Depoorter et al., 2016), whereas *Bacillus* can produce a variety of antimicrobial peptides and bacteriocins. At the level of RNA expression (Fig. 1b), at both WWTPs, the same dominant bacteria similar to those revealed by DNA-based analysis were shown, indicating the active metabolism of *Pseudomonas*, *Burkholderia*, *Bacillus* and *Streptomyces* in activated sludge. The considerable abundances and expression of these microbes indicated that they were important antibiotic producers.

3.2. Relative abundance of genes involved in antibiotic synthesis

To explore the genetic potential of antibiotic synthesis in biofilms and floc sludges, the functional gene annotation of metagenomic data against the KEGG and SEED databases was compared (Fig. S2). A total of 13 items related to antibiotic synthesis were obtained according to KEGG database, and the top abundance 8 items were streptomycin biosynthesis, monobactam biosynthesis, polyketide sugar unit biosynthesis, tetracycline biosynthesis, acarbose and validamycin biosynthesis, biosynthesis of ansamycins, penicillin and cephalosporin biosynthesis, biosynthesis of vancomycin group antibiotics. A total of 3 items were

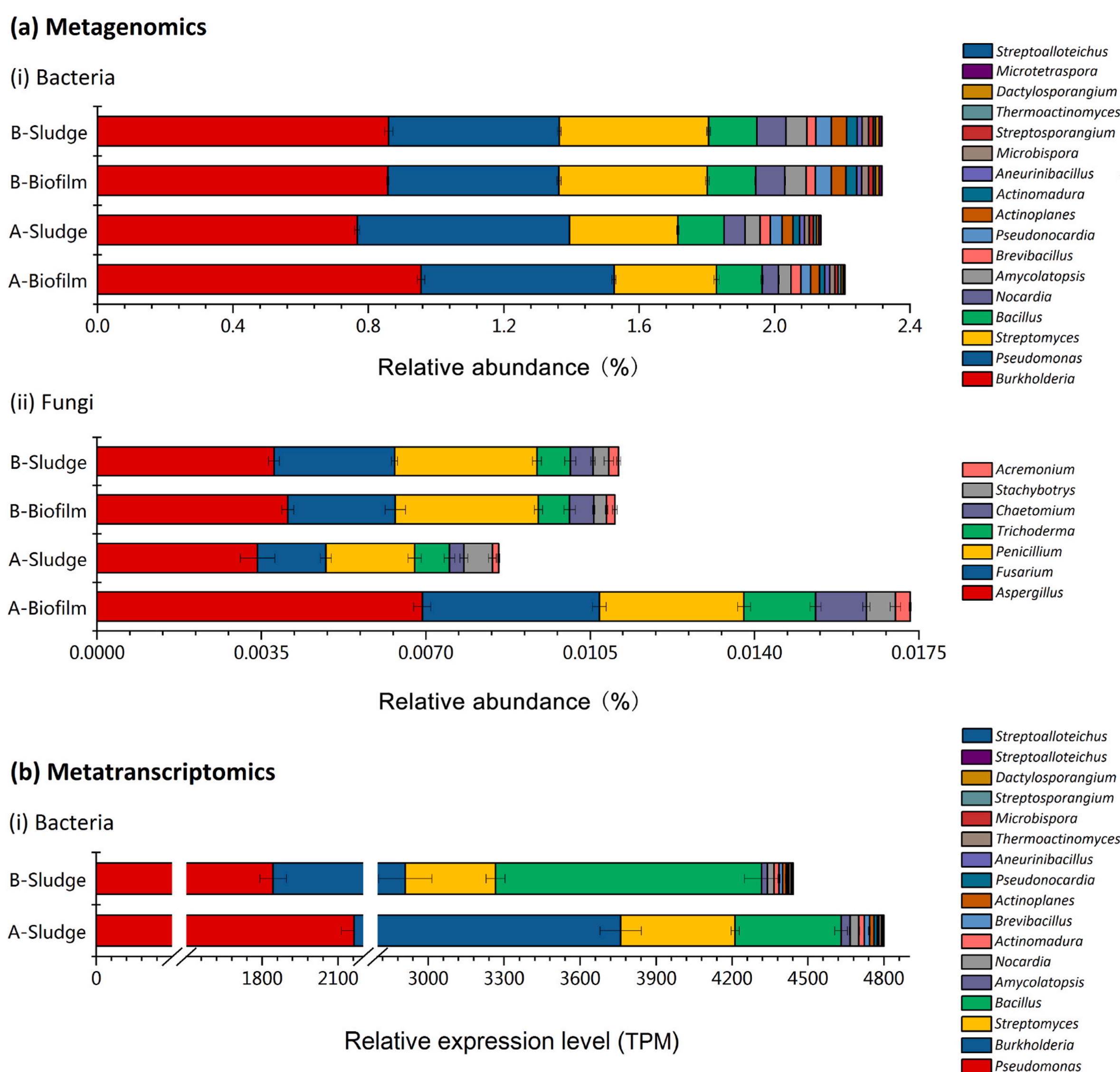

Fig. 1. The known antibiotic producers based on taxonomic profiling of **(a)** metagenomics and **(b)** metatranscriptomics, where the known antibiotic producers including (i) bacteria and (ii) fungi.

obtained according to SEED database, namely clavulanic acid biosynthesis, polymyxin synthetase gene cluster in *Bacillus* and bacilysin biosynthesis.

Antibiotic production capacities of activated sludge were evaluated by the relative abundance of ABGs. 93 ABGs belonging to 26 antibiotics classified in aminoglycosides, β-lactams, ansamycins, peptides, macrolides and other, were annotated and are shown in Fig. 2. The results showed that the relative abundances of ABGs were different between the two WWTPs and between biofilm and floc sludge, which reflected the diversity of the community structure of antibiotic producers. The relative abundances of detected ABGs had a large interval, which ranged from 0.000006% to 0.042%. The synthetic genes of β-lactams including penicillin & cephalosporin, nocardicin and clavulanic acid were detected (Fig. 2a). Among them, high abundance (>0.004%) of *cefD* (isopenicillin-N epimerase) involved in the pathway of cephalosporin C biosynthesis and can catalyze conversion of isopenicillin N to penicillin N.

Ansamycin has antituberculous and antitumor activity, and their synthetic gene abundances were different in the two WWTPs (Fig. 2b). The streptomycin synthetic genes *strI*, *strS, strK, strB1, aphD, HK* and *stsE* were detected, interestingly, abundant genes (*rfbA/rffH*, *rfbB/rffG*, *rfbC/rmlC* and *rfbD/rmlD*) involved in streptomycin synthesis were observed according to KEGG orthology (Fig. 2c and Fig. S3). These genes may provide precursors for the streptomycin biosynthesis through metabolic crossing-feed in activated sludges (Moitinho-Silva et al., 2017; Pande et al., 2015; Soto-Martin et al., 2020).

Fig. 2d shows the relative abundances of macrolides ABGs. Some of them including pimaricin, candicidin, epothilone, amphotericin & nystatin, together with fengycin and pyrrolnitrin were antifungal antibiotics. A-Biofilm harbored the most abundant synthesis genes of antifungal antibiotics. This could be attributed to the highest abundance of fungi in A-Biofilm (Fig. S4). Some study showed that the co-existence of bacteria and fungi potentially causing antagonistic interactions (Baudy et al., 2021), while a higher abundance fungi may trigger bacteria to produce more antifungal substances. The microorganisms in biofilm are closely aggregated, and the long-term production of antibiotics is easy to affect the resistance of the surrounding bacteria (Zhang et al., 2019). The previous studies have found that the reduction rate of erythromycin was low (Zhang and Li, 2011; Su et al., 2021). And in this work, high abundance of *eryA*, *eryBVI* and *eryC1* were observed, suggesting the possible presence of erythromycin synthesis.

Fig. 2e and f shows the relative abundances of gene involved in

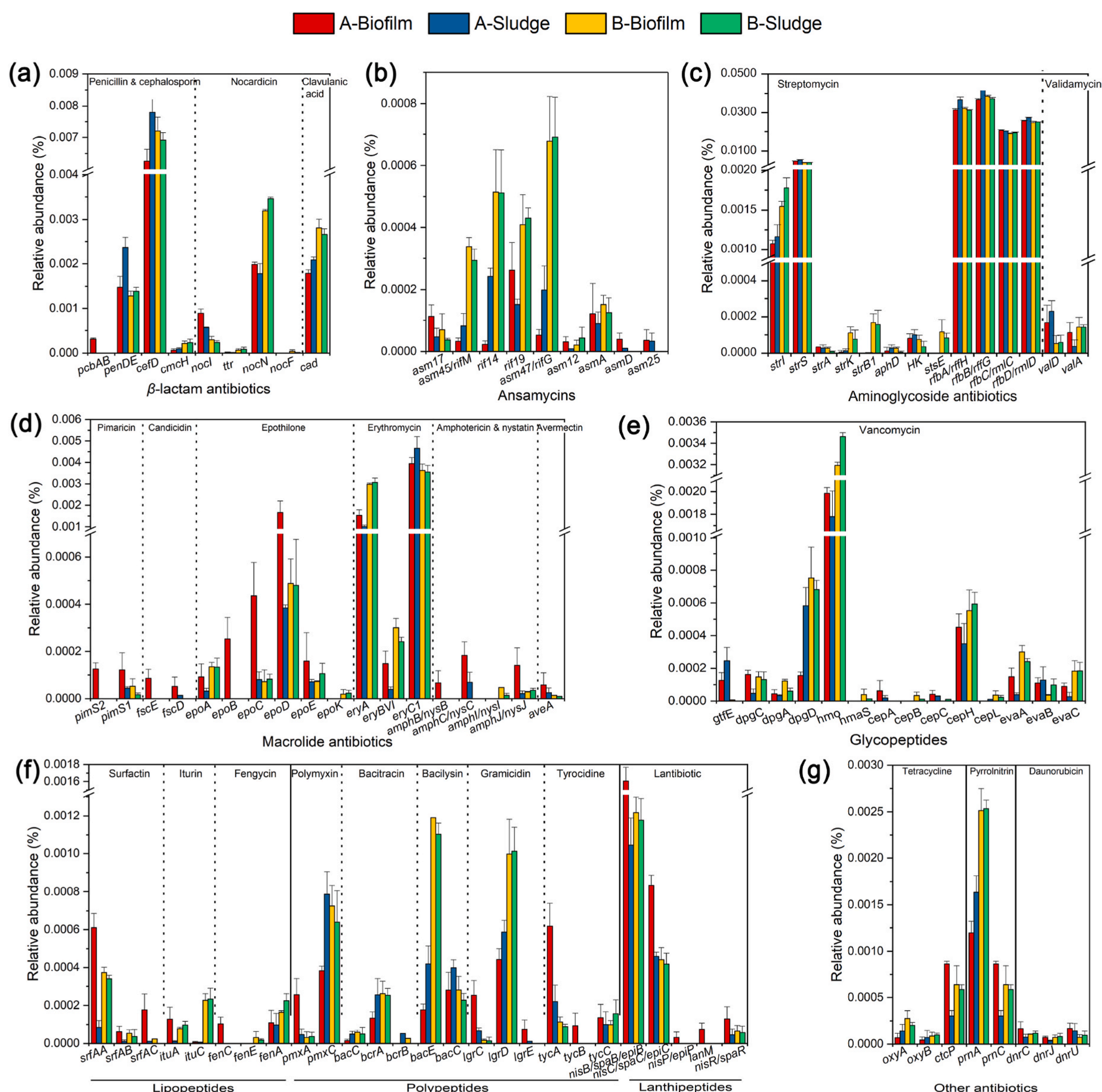

Fig. 2. The abundance of ABGs in floc sludge and biofilm of WWTP-A and WWTP-B.

synthesis of glycopeptide (vancomycins), lipopeptide (surfactin, iturin and fengycin) and polypeptide (polymyxin, bacitracin, bacilysin, gramicidin and tyrocidine), and lanthipeptide (lantibiotic) antibiotics. The biosynthesis of vancomycin divides into three steps (Xu et al., 2014), and the ABGs of vancomycin are shown in Fig. 2e. Compared with WWTP-A, WWTP-B had greater content of *evaABC*, which are responsible for dTDP-L-vancosamine synthesis. Similar with ansamycin, the ABG abundances of lipopeptides in floc sludge of WWTP-B were higher than those of WWTP-A. Lantibiotic are classified into type-A and type-B, and they have wide antibacterial spectrum and high activity. The synthetic genes of type-A including nisin, subtilin and epidermin were determined, and their abundances in biofilm were higher than those in sludge (Fig. 2f). Tetracycline synthetic genes were also detected and are shown in Fig. 2g. *oxyA* and *oxyB* involved in the polyketide skeleton sythesis of tetracycline, whereas *ctcP* is responsible for chlorination of tetracycline (Petkovic et al., 2017). A research also exhibited that the concentration of tetracyclines was increased greatly after activated sludge process in multiple WWTPs (Sabri et al., 2020). In addition, affluent ABG of pyrrolnitrin was observed, which was consistent with the high abundance of *Pseudomonas* and *Burkholderia*.

3.3. Expression of genes involved in the antibiotic biosynthesis based on metatranscriptomics

An antibiotic synthesis gene expression profile study was also carried out to speculate on the synthesis of antibiotics in the samples (Fig. 3). The two major types with high relative expression levels available according to the KEGG database were β-lactams and aminoglycosides, respectively. The results showed that the expression levels were quite different between the two WWTPs, with significantly higher expression of ABGs in WWTP-A than in WWTP-B, which is consistent with the metatranscriptomic results detected for known antibiotic producers at both sites.

The result showed the relative expression of β-lactam ABGs which include monobactams, penicillin & cephalosporin and nocardicin. The ABGs with higher abundance in the metagenomic analysis were expressed (*cefD*, *cah*, *nocI*, *nocN*), especially the ABGs of penicillin & cephalosporin could reveal a fairly complete synthetic pathway. The highest relative abundance of these genes, *cefD* (isopenicillin-N cyclase), was only expressed in small amounts, instead the gene *pac*, which finally degrades penicillin, was expressed at a higher level. This could be due to the complex microbial environment where gene expression receives many factors on the one hand, and the effect of penicillin in the influent water of the WWTPs on the other hand (Mirzaei et al., 2019). Fig. 3 also shows the expression of the genes involved in streptomycin synthesis. The genes with high relative expression (*rfbA/rffH*, *rfbB/rffG*, *rfbC/rmlC and rfbD/rmlD*) serve precisely to provide the precursor materials for streptomycin synthesis, allowing the complete synthesis of streptomycin in activated sludge.

3.4. ARGs deciphering in WWTPs

Metagenomic analysis provided detailed information about the ARGs in the two WWTPs. A total of 71 ARGs types were detected from 12 samples, and the most abundant 33 ARG types is shown in Fig. S5(a). The ARGs abundances in floc sludge and biofilm samples were unvaried notably, especially for WWTP-B. Macrolide, vancomycin, and bacitracin resistance genes were the three dominant types in all samples, followed by tetracycline, chloramphenicol, penicillin and multidrug resistance genes, which were similar to the results of previous studies (Wang et al., 2020). *macB* was the most abundance of macrolide ARGs at 1.2×10^4 copies/L, followed by *bcrA* at 0.56×10^4 copies/L, *pbp1a* at 0.12×10^4 copies/L, *vanSD* at 0.12×10^4 copies/L and *tetPB* at 0.11×10^4 copies/L (Fig. S6). The highly abundant antibiotic resistance gene types of bacitracin (*bacA*), tetracycline (*tetA, tetC, tetG, tetX*) and penicillin & cephalosporin were well expressed in the transcriptional data, similar to the metagenomic results, in addition to sulfonamide (*sul1, sul2*), streptomycin (*aph33ib*), and erythromycin (*ereA, ereB*) resistance genes. However, the most abundant macrolide resistance genes in activated sludge were only minimally expressed in WWTP-B, while vancomycins, which are second only to macrolides in abundance, were not even detected in expression. This indicates that even the highly abundant ARG may not be successfully expressed under the complex regulation of multiple factors such as genetics and environment (Hughes and Andersson, 2017). In addition, Fig. S5(b) shows that the expression of these ARGs in WWTP-A is much higher than that of WWTP-B like. The same goes for ABGs. It indicated that there might be a co-occurrence relationship between the same type of ARGs and ABGs.

Previous studies showed that a certain concentrations of antibiotics significantly promote the enrichment of ARGs (Zhang et al., 2019), and

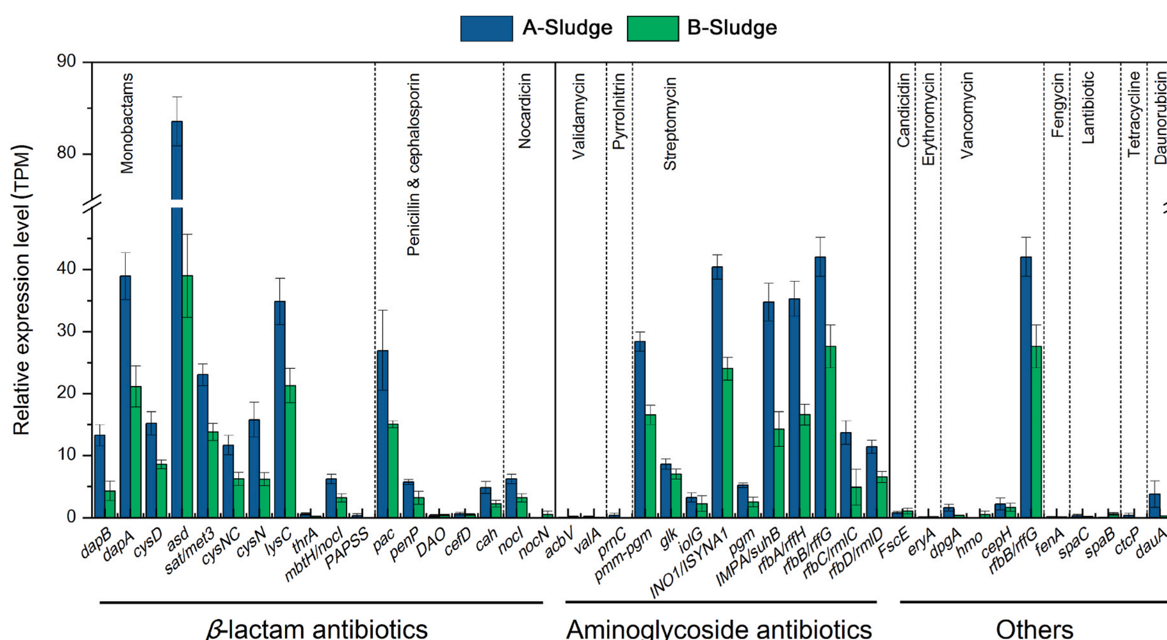

Fig. 3. Relative expression amount of ABGs in floc sludge from WWTP-A and WWTP-B.

strong antibiotic selection pressure exhibits significant effects on the diversity and abundance of ARG subtypes (Zhao et al., 2021). Apparently, these higher abundance and expression ARGs not only coincide well with the production of the corresponding antibiotic producers in WWTPs, but the associated antibiotic synthesis genes are also fully detected in the metagenomic data. Likewise, structural and statistical analyses of metagenomic data showed that the non-random co-occurrence pattern of ABGs-ARGs among the same antibiotic types was quite obvious (Fig. S7). This suggested that antibiotics synthesized by antibiotic-producing bacteria may exert sufficient selective pressure on the surrounding bacteria and show a higher incidence of ARGs-ABGs coexistence.

4. Discussion

4.1. Mechanisms of streptomycin and penicillin & cephalosporin synthesis in activated sludges

To further reveal the putative important metabolic pathways for antibiotic synthesis, the transcripts of the relevant synthetic genes were closely evaluated, and among the several items with the highest abundance associated with antibiotic synthesis obtained from the metagenomic and metatranscriptomic data, two were detected as more complete synthetic pathways, i.e., streptomycin biosynthesis and penicillins and cephalosporins biosynthesis.

Streptomycin is the first aminoglycoside antibiotic studied. The structure of this group of antibiotics is composed of aminocyclic alcohol (streptoguanidine), 6-deoxyhexose (dihydroxystreptose) and aminohexose derivatives (N-methyl-L-glucosamine). The synthesis of all these molecules is derived from glucose. The biosynthetic pathway of streptomycin based on metagenomic and metatranscriptomics data is shown in Fig. 4a. The streptomycin synthesis genes involved in the pathway for the synthesis of dTDP-L-dihydrostreptose on the right were expressed with integrity, and showed extremely high expression levels compared to the median value of all transcripts (A-Sludge: 2.43, B-Sludge: 1.43). The relative abundance of these synthetic genes in activated sludge and biofilm samples from both WWTP-A and WWTP-B did not differ significantly, whereas transcriptomic data showed that the expression in WWTP-A activated sludge was 1.5–3 times more abundant than that in WWTP-B, suggesting that they are more actively expressed in WWTP-A and the streptomycin content may be higher. The left side of the pathway for streptavidin synthesis from myo-inositol has several genes that are not annotated, probably because the streptomycin biosynthesis gene cluster has undergone a more complex process of biochemical and genetic studies, resulting in a more difficult isolation and identification of streptomycin biosynthesis intermediates (Wang et al., 2021; Mustafa

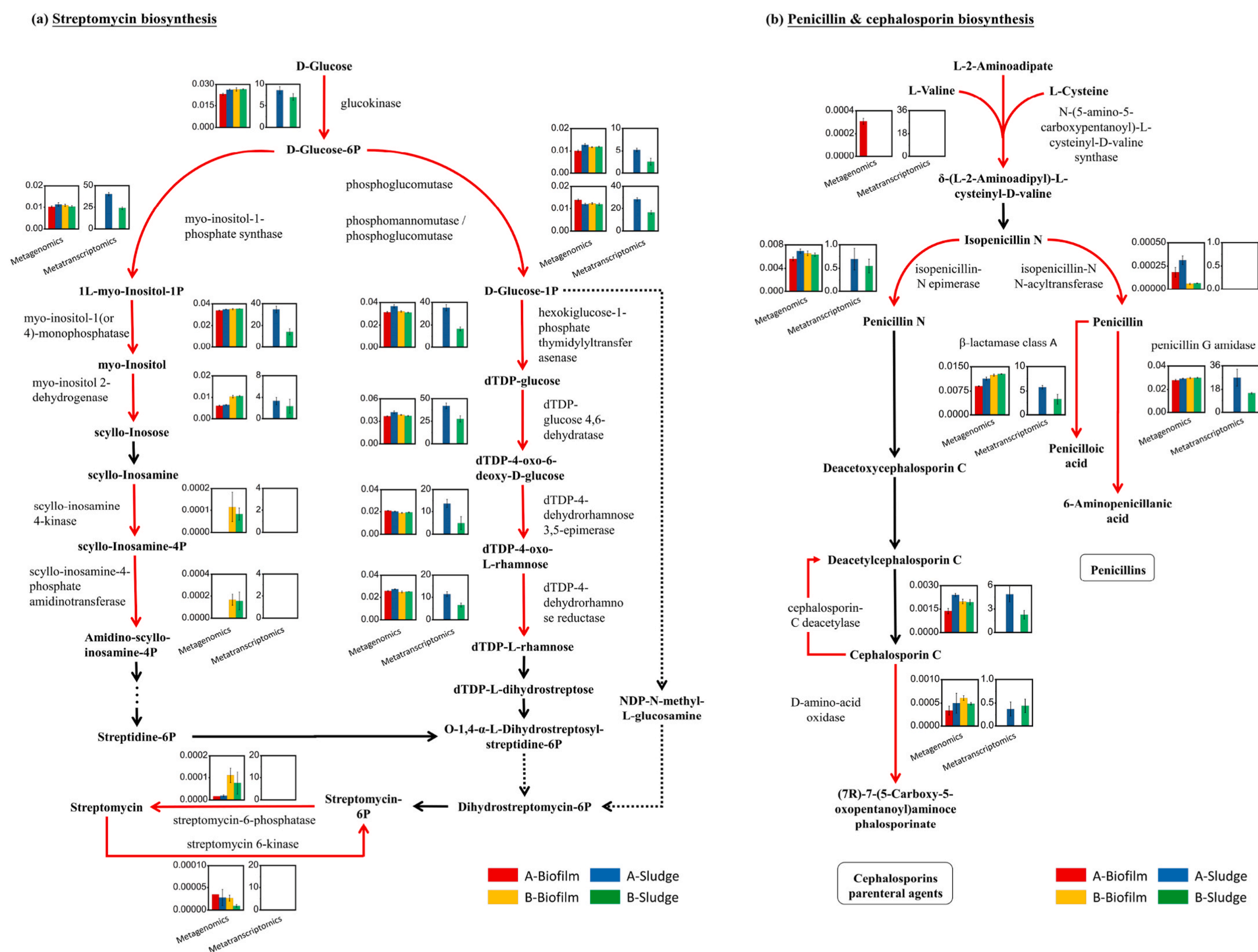

Fig. 4. Relative gene expression levels for **(a)** streptomycin biosynthesis and **(b)** penicillin & cephalosporinbiosynthesis pathways. For all graphs, left and right panels correspond to the relative abundance (%) and relative expression levels (TPM) of synthetic genes in WWTPs, respectively. Different colors represent the four sampling points. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2021). The genes ***strB1*** (scyllo-inosamine-4-phosphate amidinotransferase gene) and *stsE* (scyllo-inosamine 4-kinase gene) are involved in the biosynthesis of aminocyclic alcohols (Takano et al., 2016). A study showed the strict dependence of *strB1* on *StrR* and predicted the dependence of nine transitional units within the streptomycin biosynthesis gene cluster on *StrR* (Retzlaff and Distler, 1995; Tomono et al., 2005). *strK* finally encodes the phosphorylation of streptomycin-6-phosphate. The abundance of these three genes was low in metagenomic, and accordingly expression was not detected in the metatranscriptomics. The relatively abundant gene *strI* in the metagenomic result was also not expressed according to the metatranscriptomics, probably because the stop codon and start codon overlapped and the expression of *strI* and *strK* genes was affected by translational coupling (Mansouri and Piepersberg, 1991; Sabath et al., 2008).

Penicillin and cephalosporins are representatives of the β-lactam antibiotics. All β-lactam biosynthetic pathways share the first two steps: The intermediate tripeptide ACV is produced by α-aminoadipic acid (α-AAA), cysteine (Cys) and valine (Val) in the presence of ACVS (δ-(l-α-aminohexanediyl)-l-cysteine-d-valine synthase) encoded by the gene *pcbAB*, IPN is then produced by the isopenicillin N synthase (IPN) encoded by the gene *pcbC* (Ozcengiz and Demain, 2013). Generally, IPN in the presence of isopenicillin N acyltransferase (AT) encoded by the gene *penDE* to produce the final penicillin G (Martin et al., 1994). Combined with the metagenomic data, the transcript levels of penicillin & cephalosporin synthesis genes span a wide range, with TPM ranging from 0.372 to 29.99. The relative abundance of the genes *pcbAB* and *penDE* is low in the metagenome, and the transcription of *pcbAB*, *pcbC* and *penDE* is not only cluster copy number dependent but also repressed by glucose, resulting in their lack of expression in both WWTP-A and WWTP-B (Gutierrez et al., 1999; Nijland et al., 2010; Yang et al., 1996; Nasution et al., 2008). Nevertheless, both sampling points contain relatively high levels of genes involved in penicillin degradation (*penP*, *pac*), suggesting that penicillin is susceptible to degradation in activated sludge. Some studies have shown that penicillin and cephalosporin were undetectable in WWTPs effluent, indicating that this type of antibiotic was easily degraded (Oberoi et al., 2019). In particular, metatranscriptomic data showed that *pac*, the gene encoding penicilli G amidase, is extremely active, and the intermediate 6-aminopenicillanic acid produced by the hydrolysis of penicillin G by this enzyme can be introduced into different side chains to obtain penicillins with different potencies (Koe, 1962).

The first few synthetic steps of cephalosporin C are the same as those of penicillin. Isopenicillin N (IPN) can be regarded as a branch point of the synthetic pathway. The conversion of IPN to penicillin N in cephamycin-producing bacteria is mediated by a classical pyridoxal phosphate-dependent epimerase. This epimerization reaction is catalyzed by a two-component protein system encoded by the *cefD* that catalyzes the singling step IPN conversion to penicillin N (Ozcengiz and Demain, 2013). Modification of gene expression by directed manipulation of the *cefD1-cefD2* bidirectional promoter region can effectively facilitate the synthesis of cephalosporins (Martin et al., 2004). Notably, the relative abundance of the gene *cefD* in the metagenome was high, yet the expression level was lower than the median transcripts. The expression of gene *cah* (cephalosporin C deacetylase) was relatively more active and much higher than that of gene *DAO* encoding D-amino-acid oxidase, indicating that most of the cephalosporins synthesized by antibiotic producers were again deacetylated to remove the 3-position side chain and turned into deacetylcephalosporin C, the precursor of cephalosporin, providing favorable conditions for the next step of cephalosporin synthesis.

4.2. Analysis of the coexistence of ARGs and ABGs

The main mechanism of acquired resistance is through the horizontal transfer of ARGs from the environment or other bacteria, which is mediated by mobile genetic elements (MGEs) (Rizzo et al., 2013; Stalder et al., 2012). In the study of metagenomic sequencing to delve into the diversity and abundance of ARGs in WWTPs, a variety of highly abundant ARGs were found in activated sludge samples, as well as in abundance of MGEs, suggesting an inseparable link between the presence of MGEs and high abundance of ARGs (Guo et al., 2017). Therefore, the high abundance of ARGs based on metagenomic data might because they can be transmitted in bacteria via MGEs such as plasmids and transposons leading to increased resistance to antibiotics in activated sludge (Sabri et al., 2020). ARGs are usually located on mobile genetic elements and may be tightly linked to ABGs (Chiou and Jones, 1993). For example, the streptomycin resistance gene *strA* encoding APH(6)-Ia was identified in the streptomycin biosynthetic gene cluster of *S. griseus*. The first biosynthetic gene adjacent to *strA* is *strB1*, encoding an amidinotransferase involved in aminocyclic alcohol biosynthesis (Tolba et al., 2002). Interestingly, the simultaneous presence of *strB1* and *strA* was also detected in the metagenomic data.

It has been clearly demonstrated that microorganisms which produce a certain antibiotic are more resistant to the action of this antibiotic than species which cannot synthesize antibiotics (Demain, 1974). Many antibiotic producers have enzymes encoded by ARGs that convert their antibiotics into inactive or less active derivatives, e.g., *S. griseus* and *S. bikiniensis* synthesize streptomycin streptidinokinase along with streptomycin (Miller and Walker, 1969); *P. chrysogenum* synthesizes penicillin acylase (*pac*) (Cole, 1966). These antibiotic producers have been detected in metagenomic samples from WWTPs. And the transcriptional activity of *S. griseus* was very high. In addition, cysteine (*cysK*), a metabolite produced by antibiotic producers that inactivates antibiotics, also reaches high expression in the metatranscriptome result. All of the above indicate that antibiotic producers can both synthesize antibiotics and be resistant to them. That is, the ARGs and ABGs corresponding to the same antibiotic exist and express simultaneously in activated sludge. Coincidentally, Fig. 3 and Fig. S5(b) show extremely high expression of streptomycin synthesis genes, and the corresponding resistance genes are expressed second only to bacitracin. These implied that the expression of resistance genes is tightly linked to that of antibiotic biosynthetic genes (Mak et al., 2014). In a study of the gut microbiome of giant pandas, a co-occurrence pattern also revealed a positive association for the top ten ARGs, biosynthesis of antibiotics, and metabolic pathways. Resistant pathways annotated by KEGG in their study suggested that ARGs in the gut of giant pandas are due to the biosynthesis of antibiotics (Mustafa et al., 2021). On the basis of above results, the antibiotic synthetic microorganisms may involve in the propagation of ARGs in WWTPs and the persistent and low-level antibiotics synthesized by antibiotic producer promote the enrichment of existing ARGs.

In this work, the metagenomic and metatranscriptomic data demonstrated that the synthesis pathways of streptomycin and penicillin & cephalosporin in WWTP, suggesting that antibiotics are indeed highly likely to be synthesized in WWTP and lead to resistance in surrounding bacteria. However, it is difficult to determine the contribution of antibiotic synthetic microorganisms to the generation and propagation of ARGs due to the exogenous large variety and high concentration of antibiotics enter into the WWTPs. Therefore, in order to better investigate the production of antibiotics and consequent propagation of ARGs in the activated sludge, the future work could be focused on analyzing the dynamics of antibiotics synthesis and ARGs occurrence in the activated sludge microregion in a bioreactor without exogenous antibiotics.

5. Conclusion

The removal of antibiotics from WWTPs has been extensively studied, but the presence of antibiotics can still be detected in the effluent. The residual antibiotics in effluent can pose a potential impact on the environment. This paper raised the question of whether antibiotic synthesis is inherently present in WWTPs. The potential of antibiotic

synthesis in activated sludge and its contribution to the production of resistance genes were revealed on the basis of deciphering the distribution and expression of ABGs in activated sludge. Furthermore, this research enriches the content of the production and transmission of antibiotics and ARGs in WWTPs, and providing theoretical basis for controlling the antibiotics in activated sludge.

In this study, ABGs of 26 antibiotics were identified and their relative abundances and relative expression amount were detected. The results showed that the types and relative abundances of ABGs differed in various WWTPs and microbial aggregates. The synthetic genes for β-lactam and aminoglycoside antibiotics were most actively expressed, coinciding with the expression of the dominant antibiotic producers. From the metagenomic and metatranscriptomic data, two complete antibiotic synthesis pathways for streptomycin and penicillin & cephalosporin were obtained, and an obvious coexistence of ARGs and ABGs was also found. An indication that the antibiotics synthesized by antibiotic producer not only promote the enrichment of existing drug-resistance microorganisms, but also may be responsible for the genetic variation and gene horizontal transfer.

Credit author statement

Yu Huang: Investigation; Writing - original draft. **Kui Zou**: Writing - review & editing. **Bo Feng**: Writing - review & editing. **Tai-Ping Qing**: Writing - review & editing. **Peng Zhang**: Conceptualization; Investigation; Writing - original draft; Writing - review & editing.

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (51708475 and 21777135), Research Foundation of Education Bureau of Hunan Province, China (20B585), and the Natural Science Foundation of Hunan Province, China (2021JJ30664). The authors thank Huang Li (Shanghai Bioprofile Technology Company Ltd.) for his help with data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113741.

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