#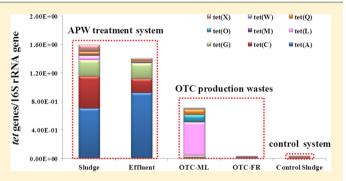 Abundance and Distribution of Tetracycline Resistance Genes and Mobile Elements in an Oxytetracycline Production Wastewater Treatment System

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S *Supporting Information*

ABSTRACT: We investigated the abundance and distribution of tetracycline resistance genes (*tet* genes) in an oxytetracycline (OTC) antibiotic production wastewater (APW) treatment system. Of sixteen *tet* genes and five mobile elements, nine *tet* genes (*tet*(A), *tet*(C), *tet*(G), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), and *tet*(X)) and two mobile elements (class 1 integron (*intI1*) and transposon Tn916/1545) were successfully quantified by real-time PCR. The relative abundance of *tet* genes in the effluent and activated sludge (1.2×10^{-4} to 1.3×10^{0}) of the APW treatment system were up to 2 orders of magnitude higher than those in the OTC fermentation residues (8.5×10^{-5} to 6.7×10^{-3}) ($P <$ 0.01), and 1−4 orders of magnitude higher than those in sewage and nonantibiotic production wastewater treatment systems (P < 0.01), showing that the discharge of abundant *tet* genes from the APW treatment system is worthy of attention. The three most abundant genes in the APW treatment system, *tet*(A), *tet*(C), and *tet*(G), exhibited significant positive correlations with *intI1* (R^2 = 0.73, 0.95, and 0.83, respectively; P < 0.05), suggesting that *intI1* may be involved in their proliferation. This is the first study showing that some measures may be required to control the discharge of antibiotic resistance genes from treated APW and activated sludge.

INTRODUCTION

Widespread antibiotic resistant bacteria and resistance genes (ARGs) have caused increasing concern worldwide,[1] leading to the regulation of antibiotic use in hospitals and clinics, as well as in agriculture and aquaculture.[2,3] Antibiotic production wastes (fermentation residues and waste mother liquors) have been listed as hazardous wastes in some countries due to the presence of high levels of residual antibiotics.[4,5] China has also prohibited the use of fermentation residues as animal food additives.[6] Waste mother liquors are normally treated using biological processes after dilution with other waste streams.[7] The high antibiotic concentrations and bacterial densities in antibiotic production wastewater (APW) treatment systems make it easy for bacteria to acquire resistance and proliferate ARGs,[8] which can be transferred among environmental bacteria and pathogens, and pose health risks to humans via various pathways.[9] Our previous study successfully isolated abundant multiantibiotic resistant bacteria harboring diverse tetracycline resistance genes (*tet* genes) from an oxytetracycline (OTC) production wastewater treatment plant.[10] However, the abundance of ARGs in treated APW and activated sludge,

which are discharged into the environment, have not yet been clarified.

As one of the most commonly used human and veterinary medicines, broad-spectrum tetracycline antibiotics are produced in large amounts around the world. *Tet* genes in the environment have been extensively studied in sewage treatment plants (STPs),[11] animal lagoons,[12] and fish farming ponds,[13] where tetracyclines have been frequently detected. Among the 44 known *tet* genes, more than 29 have been found in the environment.[10,11,14] For example, 7−13 *tet* genes have been detected in 15 STPs from different regions, with efflux protein (EFP) gene *tet*(G) the most abundant followed by *tet*(A) and *tet*(C).[11] Additionally, ribosomal protection proteins (RPP) *tet*(O), *tet*(Q), and *tet*(W) have been found the most abundant in three United States STPs,[15] while RPP *tet*(M), *tet*(O), *tet*(Q), and *tet*(W) have been detected in high abundance in animal feedlot lagoons.[12,16] In our previous study, *tet* genes

were detected in the majority of bacterial isolates from treated OTC production wastewater, with *tet*(A) and *tet*(C) being the most frequently detected.[10] The distribution of *tet* genes appears to vary among different environments, which may be associated with bacterial compositions, antibiotic levels, horizontal gene transfer, etc.[1,8,17] It is not clear, however, how *tet* genes are distributed in the APW treatment system, and what important factors affect their distribution.

The main target of this study was to understand the abundance and distribution of *tet* genes in an APW treatment system that mainly receives production wastewater from an OTC manufacturing plant in northern China. Among the 16 *tet* genes determined using PCR, 11 detected *tet* genes were quantified for all samples, including the antibiotic production waste, using real-time PCR. To understand the potential mechanisms for the distribution of *tet* genes, some important mobile elements were also quantified using real-time PCR, and the diversities of *tet*(C), *tet*(M), and *tet*(X) as well as bacterial 16S rRNA genes were analyzed by constructing clone libraries. For the first time, this study revealed the intensity and compositions of *tet* genes discharged from an APW treatment system.

■ EXPERIMENTAL SECTION

Study Facilities, Sampling Sites, and Characterization of Samples. More than 1000 tons of OTC are produced every year in the studied antibiotic manufacturing plant in Hebei Province, China. Following slab filtration and OTC extraction (SI Figure S1.a), the OTC waste mother liquor (OTC-ML) is diluted by machine washing water and a small amount of sewage within the plant to produce an average flow of 5000 m³ day⁻¹. The mixed wastewater is then treated by a biological system which includes a sequential batch reactor (SBR) and a two-stage submerged biological contact reactor using fiber bundle as the biocarrier (SI Figure S1.b). The hydraulic retention time is 8 h for each reactor. The final effluent from the APW treatment system is discharged into a nearby river. The concentration of mixed liquor suspended solids in the SBR is maintained at 4000−5000 mg/L. One cycle of the SBR includes four operational steps, namely filling (1 h), aeration (5 h), settling (1.5 h), and decanting (0.5 h). This APW treatment system has been in use since 2000.

Water samples from the APW treatment system, including the influent (W1) and the final effluent (W2), were collected with 24-h composite samples taken every 4 h four times (March and June 2009, January 2010, and January 2012). Activated sludge samples of the SBR (S1) and the biological contact reactor (S2) were collected in January 2010 and January 2012. The OTC production waste was collected from the production workshop in May 2010. The OTC fermentation residue (OTC-FR) was collected as soon as it was separated from the OTC fermentation broths. Waste OTC mother liquor (OTC-ML) was collected from its storage tanks. Samples were kept below 4 °C until pretreatment or analysis, which was conducted within 48 h. Aerobic activated sludge samples from two nonantibiotic (inosine and ethanol) production wastewater treatment plants were collected as control systems, because these facilities only receive inosine and ethanol production wastewater and sewage, respectively.

Chemical and biological characteristics and concentrations of OTC related compounds (OTC and its three hydrolysates, 4-epi-OTC, α-Apo-OTC, and β-Apo-OTC) were determined as described previously,[18,19] with related methods briefly presented in the Supporting Information. All samples were analyzed in duplicate or triplicate. The average results are summarized in SI Table S1−S2.

DNA Extraction and PCR. Ten mL of each wastewater sample was filtered through 0.22-μm polycarbonate membranes (GTTP, Millipore, Ireland), and biomass on the membranes was collected in 2-mL sterilized tubes for DNA extraction using the Proteinase K method.[20] Sludge samples were centrifuged at 10 000 rpm for 10 min at 4 °C, and 0.25 g (wet weight) of sediment for each sample was used for DNA extraction using a FastDNA SPIN kit for soil (Qbiogene, Solon OH). The volumes of DNA solutions from the water and sludge samples were 50 and 80 μL, respectively. Concentrations and quality of the extracted DNA were checked by spectrophotometric analysis on a NanoDrop ND-1000 (Nanodrop, USA) and electrophoresis on a 1% (weight/volume) agarose gel.

Sixteen *tet* genes (six RPP genes (*tet*(M), *tet*(O), *tet*(Q), *tet*(T), *tet*(W), and *tetB*(P)), nine EFP genes (*tet*(A), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(Z), and *tetA*(P)) and one inactivating enzyme *tet*(X)) and five mobile elements (Class 1 to 4 integrons (*intI1*−*intI4*) and transposon Tn916/1545) were investigated. The PCR assays for DNA extracted from the SBR activated sludge (S1) were conducted in 25-μL volume reactions using an ABI PCR System 9700 (ABI, USA). The PCR mixture consisted of 1× rTaq buffer, 5 nM dNTP, 0.625 U Ex Taq (Takara, Japan), 400 nM each primer, 0.5 mg mL⁻¹ BSA, and 1−2 μL of DNA. Primers targeting the sixteen *tet* genes and five mobile elements were selected from published literature,[21−25] with their amplification sizes and conditions listed in SI Table S3. The temperature program consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C, 30 s at different annealing temperatures and extension at 72 °C for 45 s, and finished with a final extension step at 72 °C for 7 min. Sterile water was used as the negative control in each run. The PCR products were checked by electrophoresis on a 1% (weight/volume) agarose gel in 1 × TBE buffer. The positive amplicons were further assured by cloning and sequencing.

Real-Time PCR. Eleven detected *tet* genes (*tet*(A), *tet*(C), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), and *tet*(X)), three detected mobile elements (*intI1*, *intI2*, and Tn916/1545), and the 16S rRNA gene were quantified for all samples using SYBR-Green real-time PCR. Standard plasmids carrying target genes were obtained by TA clones and extracted using a TIANpure Mini Plasmid kit (Tiangen, China).[26] Concentrations of the standard plasmids (ng μL⁻¹) were determined with the Nanodrop ND-1000 (Nanodrop, USA). Their copy concentrations (copies μL⁻¹) were then calculated by the following formula.[27]

$$\text{copy concentration (copies } \mu L^{-1})$$
$$= \frac{\text{DNA mass concentration (ng } \mu L^{-1})}{\text{DNA molecular weight (g mol}^{-1})} \times 6.02 \times 10^{23}$$
$$\times 10^{-9}$$

The 25-μL reactions typically contained 1 × Sybr Green I, 1 × Dye (Takara), 200 nM each primer, 0.5 mg mL⁻¹ BSA, and 2 μL of DNA templates. Real-time PCR was run on an ABI7300 apparatus (ABI, USA) by the following program: 95 °C for 30 s, and 40 cycles consisting of the following: (i) 95 °C for 10 s, (ii) annealing temperature for 15 s, (iii) 72 °C for 15 s, and (iv) 78 °C for 26 s to collect the fluorescent signals. The melting

process was automatically generated by the ABI7300 software. Triplicate real-time PCR assays were performed for the decimally diluted standard plasmids to obtain the standard curves. Duplicate real-time assays were performed for all samples and negative controls. To prevent the inhibition of the sample matrix, 10−100 fold diluted samples were used for quantification.

The following requirements were satisfied to obtain reliable quantification: R^2 higher than 0.99 for standard curves over 5 orders of magnitude and amplification efficiencies based on slopes between 90% and 110% (SI Table S4). The specificity was assured by the melting curves and gel electrophoresis. Gene copies per sample volume are shown in SI Table S5. To minimize the variance caused by different bacterial abundance, as well as different DNA extraction and quantification efficiencies, the relative abundance of *tet* genes and mobile elements was obtained by normalizing their copy numbers to those of the 16S rRNA genes (SI Table S6).[28]

Cloning and Phylogenetic Analysis of *tet* Genes. Clone libraries of *tet*(C), *tet*(M), and *tet*(X), which represent three different resistance mechanisms, were constructed for OTC-FR, OTC-ML, W1, W2, and S1. The target gene fragments were amplified in triplicate using the same primers as for PCR and real-time PCR. Combined amplification products of the triplicate PCR assays were purified by a Fermentas GeneJET gel extraction kit (Thermo, USA), followed by inserting into the pMD-18T cloning vector (Takara) and transforming into JM109 *Escherichia coli* (Takara). Transformants were further selected by blue−white screen with X-Gal and confirmed by PCR with plasmid vector-specific primers M13F and M13R using 55 °C as the annealing temperature. Positive clones were randomly selected for sequencing on an ABI3300 sequencing machine (ABI, USA). Sequences were subjected to homology analysis using the software Lasergene version 7 (DNAStar, USA). Sequences that exhibited more than 98% identity to each other were considered as one type, from which only one representative sequence was chosen to construct the phylogenetic trees. Online similarity searching was performed using the NCBI BLAST tool, and the most similar GenBank sequences were included in the neighbor-joining trees using MEGA version 4.1 (http://megasoftware.net/). Bootstrap analysis with 1000 replicates was used to evaluate the significance of the nodes. The sequences of *tet*(M) obtained in this study were deposited in the European Nucleotide Archive gene bank under Accession number HE580463 to HE580643, and the sequences of *tet*(C) and *tet*(X) were deposited in the NCBI GenBank under Accession number JQ668333 to JQ668515.

Cloning and Phylogenetic Analysis of the 16S rRNA Genes. Clone libraries of the 16S rRNA genes were constructed for OTC-FR, OTC-ML, W1, W2, and S1 using primers 27F and 1492R by the same procedure as the above *tet* genes.[29] Classification of the operational taxonomic units (OTUs), and calculation of coverage and Shannon index were carried out using Dotur software.[30] The 16S rRNA gene sequences obtained in this study were deposited in the NCBI GenBank under Accession number JN245629 to JN245879.

Data Analysis. All statistical analyses were performed using SPSS version 15.0. Paired samples *t* test was used to assess the significance of the differences between different systems and samples based on *P*-values. Pearson's bivariate correlation analysis was used to assess the relation among *tet* genes and mobile elements.

■ RESULTS

Concentrations of OTC Related Compounds. As summarized in SI Table S2, the total concentrations of OTC related compounds (OTC and its three hydrolysates) were extremely high in the OTC fermentation residues (OTC-FR) (44 102.7 mg (kg dry weight)$^{-1}$) and waste OTC mother liquors (OTC-ML) (1074.9 to 1479.8 mg L^{-1}). The total concentrations were 1.14−12.36 mg L^{-1} in the SBR influent (W1) and 0.36−2.35 mg L^{-1} in the final effluent (W2) and were as high as 40.7−170.2 mg (kg dry weight)$^{-1}$ in the activated sludge (S1 and S2), showing the adsorption of antibiotics by biomass.

Abundance of *tet* Genes and Mobile Elements. Among the sixteen *tet* genes and five mobile elements examined by PCR, eleven *tet* genes (*tet*(A), *tet*(C), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), and *tet*(X)) and three mobile elements (*intI1*, *intI2*, and Tn916/1545) were detected in the SBR activated sludge sample (S1) collected in March 2009. These *tet* genes and mobile elements were then quantified by real-time PCR for the OTC production waste and samples from the APW treatment system.

Except for *tet*(E), *tet*(K), and *intI2*, all other genes were detected by real-time PCR in all samples. As shown in SI Table S5, the highest abundance of *tet* genes and mobile elements per sample volume was observed in the sludge samples (S1 and S2), where individual *tet* genes ranged from 5.7×10^6 to 6.7×10^9 copies/mL with the total *tet* genes (*tet*^{T}) at the level of 10^{10} copies/mL, and mobile elements ranged from 2.2×10^6 to 1.6×10^{10} copies/mL. The gene abundance per sample volume in the APW treatment system (W1, W2, S1, and S2) was significantly higher than those in the OTC production waste (OTC-FR and OTC-ML) ($P < 0.05$). Since the biomass contents could affect the gene abundance,[12] relative abundance of *tet* genes and mobile elements (gene copies per 16S rRNA gene) was used for further analyses (Figure 1, SI Figure S2, and SI Table S6).
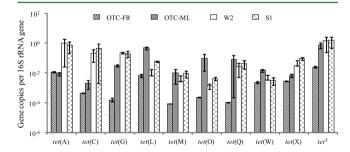

Figure 1. Relative abundance of *tet* genes (gene copies per 16S rRNA gene) in samples from the OTC production and APW treatment systems. *tet*^{T} is the total relative abundance of the 9 *tet* genes. Columns are the mean values of 2−4 sampling campaigns. Error bars represent the standard deviations.

In OTC-FR, the relative abundance of individual *tet* genes was in the range of 8.5×10^{-5} to 1.0×10^{-2}, with *tet*^{T} at 2.4×10^{-2}. In OTC-ML, the relative abundance of individual *tet* genes was in the range of 2.7×10^{-4} to 3.6×10^{-1}, with *tet*^{T} at 7.1×10^{-1} (SI Table S6). *tet*(A), *tet*(C), and *tet*(G) were the three most dominant *tet* genes in the effluent and activated sludge of the APW treatment system (W2, S1, and S2), ranging from 7.2×10^{-2} to 1.7×10^0, followed by *tet*(X) at the level of 2.9×10^{-2} to 1.0×10^{-1} (Figure 1, SI Table S6). Compared to the relative abundance in influent, *tet*(G) increased in the

effluent while *tet*(L), *tet*(M), *tet*(Q), and *tet*(W) decreased ($P <$ 0.05), with other genes and the total *tet* genes (tet^T) showing no significant change after treatment ($P > 0.05$) (SI Figure S2).

As shown in SI Table S6, the relative abundance of *intI1* was about 1.1×10^{-1} in OTC-ML, higher than that in OTC-FR (3.5×10^{-4}). The relative abundance of *intI1* was 2.2×10^{-1} to 3.2×10^{0} in the effluent (W2) and sludge samples (S1 and S2) of the APW treatment system. In addition, Tn916/1545 was 1.7×10^{-6} and 1.0×10^{-1} in OTC-FR and OTC-ML, respectively, and 4.9×10^{-4} to 1.5×10^{-2} for samples from the APW treatment system. Similar to a previous study,[28] Tn916/1545 showed a positive correlation with *tet*(M) during treatment ($R^2 = 0.48$, $P < 0.05$, SI Figure S3), indicating that Tn916/1545 may be responsible for the transfer of *tet*(M).

Cloning and Phylogenetic Analysis of *tet*(C), *tet*(M), and *tet*(X). Clone libraries of *tet*(C), *tet*(M), and *tet*(X) were constructed for OTC-FR, OTC-ML, W1, W2, and S1. BLAST searches in the NCBI GenBank database confirmed that all the sequenced clones matched known *tet*(C), *tet*(M), and *tet*(X) genes, with identities between 98% and 100%. The phylogenetic trees of *tet*(C), *tet*(M), and *tet*(X) are shown in SI Figures S4−S6. The distributions of *tet*(C) and *tet*(M) are shown in SI Table S7 and Table 1, respectively.

Table 1. Distribution of Phylogenetic Groups among *tet*(M) Clone Libraries for the Samples from the OTC Production and APW Treatment Systems

type	percentage of each type to total no. of clones (%)				
	OTC-FR	OTC-ML	W1	W2	S1
Type 1	97.2	71.4	81.6	23.3	38.1
Type 2	2.8	20.0	8.9	-[a]	2.4
Type 3	-	-	-	3.3	-
Type 4	-	-	-	3.3	-
Type 5	-	8.6	10.5	66.7	59.5
Type 6	-	-	-	3.3	-
NO. of clones	36	35	38	30	42

[a]"-": absent.

The *tet*(C) sequences were divided into 4 types (SI Figure S4). Type 1, which was identical to the *tet*(C) sequences in *Escherichia coli* (EU751610−EU751613), *Escherichia coli* plasmid (Y19114), and *Francisella tularensis* plasmid (NC_002109), was the most dominant type for all samples. Type 2 was found in OTC-FR and OTC-ML at the frequency of 19.0% and 13.0%, while Type 3 was found in OTC-FR at the frequency of 4.8%, and Type 4 was found in OTC-ML at the frequency of 4.3% (SI Table S7).

The *tet*(M) clones were sorted into six types (Type 1−Type 6) according to the homology analysis (SI Figure S5). As shown in Table 1, Type 1, which was identical to the *tet*(M) sequences found in various plasmids, conjugative transposons, and chromosomes of both gram-negative and gram-positive bacteria, was dominant (81.6%−97.2%) in the clone libraries of OTC-FR, OTC-ML, and W1, but was less abundant in W2 (23.3%) and S1 (38.1%). Type 5, which was 99% identical to the *tet*(M) sequences in *Streptococcus agalactiae* (GU318219), *Streptococcal* conjugative shuttle transposon Tn1545 (X04388), and *Streptococcus pneumoniae* partial transposon Tn1545 (AM889142), became dominant in W2 and S1 (59.5%). Types 2, 3, 4, and 6 were detected with relatively low abundance (2.4%−20.0%).

The *tet*(X) sequences in OTC-ML, W1, W2, and S1 showed a very conservative type identical to those in *Sphingobacterium* (EU864422) and *Bacteroides* Transposon Tn4351 (M37699) (SI Figure S6). Cloning of *tet*(X) was not obtained for OTC-FR.

Cloning and Phylogenetic Analysis of the 16S rRNA Genes. Clone libraries of the 16S rRNA genes were constructed for OTC-FR, OTC-ML, W1, W2, and S1, with 40−68 clones sequenced for each sample (SI Table S8). *Streptomyces*, which has been used as OTC production bacterium,[31] was dominant (61.5%) in OTC-FR, but disappeared in the subsequent samples (OTC-ML, W1, W2, and S1). Class *Bacilli* was abundant in OTC-FR (33.3%) and OTC-ML (63.4%). In the influent (W1), classes *Clostridia* and *Bacteroidetes* accounted for 27.4% and 21.9% of the total clone numbers, followed by *Gammaproteobacteria* and *Bacilli* (16.4% and 11.0%, respectively). In the effluent (W2), *Betaproteobacteria* was the most abundant (44.3%) followed by many unclassified bacteria (24.6%). For the activated sludge (S1), Class *Betaproteobacteria* was dominant (42.6%) followed by *Bacteroidetes* (25.9%). The distribution of gram-positive (G+) and gram-negative (G−) bacteria in all 16S rRNA gene clone libraries is summarized in Table 2. The G+ bacteria were dominant in OTC-FR (97.4%) and OTC-ML (73.2%), while G− bacteria became dominant in W2 (67.2%) and S1 (100%).

Table 2. Distribution of Gram-Positive and Gram-Negative Bacteria among the 16S rRNA Gene Clone Libraries for the Samples from the OTC Production and APW Treatment Systems

bacterial group	percentage of Clones (%)				
	OTC-FR	OTC-ML	W1	W2	S1
gram-positive	97.4	73.2	42.6	6.9	-[a]
gram-negative	2.6	26.8	51.5	67.2	100.0
unclassified	-	-	5.9	25.9	-

[a]"-": absent.

■ DISCUSSION

Worldwide annual output of the tetracycline class of antibiotics has reached more than 20 000 tons.[32] During OTC production, waste mother liquor containing OTC related compounds at concentrations of over 1000 mg L^{-1} is generated and treated with biological processes after dilution.[7] As summarized in SI Table S1, high levels of OTC related compounds were observed in the wastewater (0.36−12.36 mg L^{-1}) and activated sludge samples (40.5−170.2 mg (kg dry weight)$^{-1}$),[33] which were much higher than those previously reported in other environments, such as STPs (0.06−1.10 μg L^{-1}),[34] hospital wastewater (<100 μg L^{-1}),[35] animal feces (1.15−145.75 μg L^{-1}),[36,37] and soils adjacent to swine farms (5.36−377.77 μg (kg dry weight)$^{-1}$).[32]

It is well accepted that ARGs are selected and enriched by antibiotic residues.[8] SI Table S9 compares the relative abundance of *tet* genes in the effluent and sludge from the APW treatment system with those from STPs and the control systems receiving inosine and ethanol production wastewater. The relative abundance of *tet* genes in the activated sludge from the APW treatment system was 1−4 orders of magnitude higher than those in STPs and the control systems ($P <$ 0.01).[11,38−40] This comparison clearly shows that high fluxes of

tet genes could be released into the environment through the discharge of treated effluents and the disposal of excess sludge from an APW treatment system.

Antibiotic fermentation residues and waste mother liquors have been listed as hazardous wastes in some countries, and antibiotic fermentation residues have been prohibited as animal food additives in China since 2002.[4−6] Our results (Figure 1) showed that the effluent and activated sludge from the APW treatment system contained higher levels of *tet* genes than the OTC fermentation residues ($P < 0.01$), and had no significantly different gene levels from the OTC mother liquors ($P > 0.05$). There are indications that *tet* genes may be able to spread from environmental bacteria to pathogens after being discharged into the environment.[10] Thus health risks posed by the discharge of treated APW and activated sludge should be evaluated from the aspect of ARGs, and proper regulations to manage these two release routes are required to control the proliferation of ARGs in the environment.

In the activated sludge of the APW treatment system, efflux protein (EFP) genes *tet*(A), *tet*(C), and *tet*(G) were the most abundant, followed by the enzymatic modification gene *tet*(X) (SI Table S6). Zhang et al. reported that, among thirteen *tet* genes, *tet*(G) was the most abundant followed by *tet*(A) and *tet*(C) in ten out of fifteen STPs, while *tet*(A) and *tet*(C) were more abundant in the other five STPs.[11] Our former study has also shown that *tet*(A) and *tet*(C) were the most frequently detected *tet* genes in bacteria isolated from the same APW treatment system (69.3% and 42.9%, respectively),[10] where *tet*(G) was not detected, perhaps due to the use of a different primer set.[22] Thereby, EFP genes *tet*(A), *tet*(C), and *tet*(G) could prevail in wastewater treatment systems with different levels of residual antibiotics. However, RPP genes *tet*(O), *tet*(Q), and *tet*(W) were found to be the most abundant *tet* genes in three American STPs.[15] Research conducted in animal feedlot lagoons found that RPP genes *tet*(M), *tet*(O), *tet*(Q), and *tet*(W) were more abundant than EFP genes *tet*(B), *tet*(C), and *tet*(L).[12,16] Hu et al. only detected *tet*(A), *tet*(B), and *tet*(M) among twenty *tet* genes in tetracycline resistant *Escherichia coli* isolated from a natural river.[41] Thus, the different distribution patterns of *tet* genes in different environments may be associated with bacterial compositions, antibiotic levels, horizontal gene transfer, etc.[1,8,17] As will be discussed below, the dominance of *tet*(A), *tet*(C), and *tet*(G) in our study was likely related with the host compositions and their potential transfer ability, both of which could be affected by the high antibiotic concentrations in the APW treatment system.

Generally, G+ bacteria mainly harbor RPP *tet* genes and a few EFP *tet* genes including *tet*(K), *tet*(L), *tet*(Z) and *tet*(T), while G− bacteria harbor both EFP and RPP *tet* genes, with EFP *tet* genes dominating.[17] In the present study, *tet*(L) was the most abundant gene in OTC-ML, which may be related to the dominance of G+ bacteria (73.2%). On the other hand, the dominance of G− bacteria in the effluent and activated sludge (Table 2) may have allowed EFP genes *tet*(A), *tet*(C) and *tet*(G) to prevail and even increase during the APW treatment. The relative abundance of total *tet* genes was low (10^{-2}) in OTC-FR, which was dominated with *Streptomyces* and *Bacilli* (SI Table S8). As a production bacterium, *Streptomyces* may have different self-protection mechanisms, resulting in low abundance of *tet* genes.[42,43]

Horizontal gene transfer, which could be carried out by mobile elements, including integrons, conjugative transposons,

and plasmids, is a very important pathway for ARGs' proliferation, especially under antibiotic selection pressure.[16,44,45] As shown in SI Figure S7, *tet*(A), *tet*(C), and *tet*(G) exhibited significant positive correlations with *intI1* (R^2 = 0.84, 0.95, and 0.83, respectively; $P < 0.05$). In our previous study, *tet*(A), *tet*(C), and *intI1* were detected with high frequencies in antibiotic resistant bacteria isolated from the same APW treatment plant.[10] As the most common integron, *intI1* is reported to integrate and express more than 100 types of resistance genes by gene cassettes, most of which are aminoglycoside and trimethoprim resistance genes and β-lactamases.[46] Although no *tet* genes have yet been detected in any gene cassette of *intI1*, some studies have shown that *intI1* can accrete noncassette resistance genes outside the cassette region by the "insertion sequence common region" (ISCR), a newly highlighted complex integron element usually linked to *intI1*.[47] Previous findings have revealed that *intI1* coexists with *tet*(A) and *tet*(C) in conjugative plasmids by which they can be easily cotransferred into recipients.[48,49] Moreover, *tet*(A) and *tet*(G) have been found on ISCR2 and ISCR3, the latter of which is linked to *intI1*.[47] Thus, *intI1* may play certain roles in the transfer of *tet*(A), *tet*(C), and *tet*(G), resulting in the dominance and increase of these genes in the APW treatment system.

According to the sequences from the NCBI GenBank, *tet*(M) has diverse types, which makes it suitable for source tracking of *tet* genes.[23,50] As shown in Table 1, OTC-FR, OTC-ML, and W1 shared similar distribution of *tet*(M) types, with Type 1 dominant, while W2 and S1 shared a similar pattern, with Type 5 dominant. The distribution patterns of *tet*(M) types in different samples suggested that some *tet* genes in the influent may originate from the OTC producing wastes, probably by gene transfer, while others occurred in the effluent and sludge under the selection pressure of high antibiotic concentrations. As for *tet*(C), Type 1 detected in all samples at high proportions was identical to the known sequences obtained from the NCBI GenBank, which were conservative based on homology analysis. New types of *tet*(C) (Types 2−4) only occurred at low frequencies in OTC-FR and OTC-ML, where the antibiotic selection pressure was extremely strong.

tet(X) encodes an NADP-dependent monooxygenase that catalyzes the degradation of tetracyclines.[51] *tet*(X) was found at low levels in the influent, but showed a high abundance in the effluent and activated sludge of the APW treatment system (SI Table S6). The sequences of *tet*(X) in the APW treatment system were 100% identical to those found in the two reported host bacteria *Bacteroides* and *Sphingobacterium*.[52,53] Although OTC is considered difficult to biodegrade,[32,54] the high abundance of *tet*(X) in the activated sludge suggests that *tet*(X) may have been selected for some unknown function under the high residual OTC levels, which warrants further study.

This study revealed for the first time that the relative abundance of *tet* genes and mobile elements in treated effluent and sludge from the APW treatment system was higher than those in the OTC fermentation residues which have been listed as hazardous wastes ($P < 0.01$), and higher than STPs and the control systems ($P < 0.01$), making it essential to manage these routes in relation to ARGs. The dominance of the EFP genes *tet*(A), *tet*(C), and *tet*(G) in the APW treatment system was related with the bacterial community compositions and the presence of *intI1*. Further studies are necessary to explore the

proliferation pathways of ARGs under high antibiotic selection pressure in APW treatment systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Flowchart and sampling sites of the OTC production wastes (Figure S1.a) and the APW treatment system (Figure S1.b); Relative abundance of *tet* genes and *intI1* (gene copies per 16S rRNA gene) in the influent (W1) and effluent (W2) (Figure S2); Correlation between the relative abundances of *tet*(M) and Tn916/1545 (Figure S3); Neighbor-joining phylogenetic tree of representative *tet*(C) gene sequences (Figure S4); Neighbor-joining phylogenetic tree of representative *tet*(M) gene sequences (Figure S5); Neighbor-joining phylogenetic tree of representative *tet*(X) gene sequences (Figure S6); Correlations between the relative abundance of *tet*(A), *tet*(C), *tet*(G) and *intI1* (gene copies per 16S rRNA gene) for samples from the APW treatment system (Figure S7); Chemical and biological characteristics of the wastewater (Table S1); Concentrations of OTC and its hydrolysates in wastewater (Table S2); Primers, PCR conditions used in this study (Table S3); The detection limit, standard curve, and amplification efficiency of Sybr-Green real-time PCR (Table S4); Copies of *tet* genes per sample volume (Table S5) and per 16S rRNA gene (Table S6); Distribution of phylogenetic groups among *tet*(C) clone libraries (Table S7); Distribution of phylogenetic groups among 16S rRNA clone libraries (Table S8); Comparison of the relative abundance of *tet* genes and mobile elements in the effluent and activated sludge from the APW treatment system and those from STPs and control systems (Table S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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