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Antibiotic Resistance Characteristics of Environmental Bacteria from an Oxytetracycline Production Wastewater Treatment Plant and the Receiving River▽†

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We characterized the bacterial populations in surface water receiving effluent from an oxytetracycline (OTC) production plant. Additional sampling sites included the receiving river water 5 km upstream and 20 km downstream from the discharge point. High levels of OTC were found in the wastewater (WW), and the antibiotic was still detectable in river water downstream (RWD), with undetectable levels in river water upstream (RWU). A total of 341 bacterial strains were isolated using nonselective media, with the majority being identified as *Gammaproteobacteria*. The MICs were determined for 10 antibiotics representing seven different classes of antibiotics, and the corresponding values were significantly higher for the WW and RWD isolates than for the RWU isolates. Almost all bacteria (97%) from the WW and RWD samples demonstrated multidrug-resistant (MDR) phenotypes, while in RWU samples, these were less frequent (28%). The WW and RWD isolates were analyzed for the presence of 23 tetracycline (*tet*) resistance genes. The majority of isolates (94.2% and 95.4% in WW and RWD, respectively) harbored the corresponding genes, with *tet*(A) being the most common (67.0%), followed by *tet*(W), *tet*(C), *tet*(J), *tet*(L), *tet*(D), *tet*(Y), and *tet*(K) (in the range between 21.0% and 40.6%). Class I integrons were detected in the majority of WW and RWD isolates (97.4% and 86.2%, respectively) but were not associated with the *tet* genes. We hypothesize that the strong selective pressure imposed by a high concentration of OTC contributes to the wide dissemination of tetracycline resistance genes and other antibiotic resistance genes, possibly through mobile genetic elements.

The widespread emergence of antibiotic resistance, particularly multidrug resistance (MDR), among bacterial pathogens has become one of the most serious challenges in clinical therapy (22, 46). Some pathogens, such as MDR *Klebsiella pneumoniae* and *Acinetobacter baumannii*, are now virtually untreatable with current antibiotics (14, 30). Acquisition of resistance genes through horizontal transfer has been found to be ubiquitous in clinical pathogens (22). Environmental bacteria have been shown to be a reservoir of antibiotic resistance genes and a potential source of novel resistance genes in clinical pathogens (10, 12). Horizontal transfer of genes between bacterial strains could be facilitated by mobile genetic elements, such as plasmids, transposons, bacteriophages, integrons, insertion elements (IS), and genomic islands (13). Some elements, including class I integrons, conjugative plasmids, and transposons, are frequently linked to antibiotic resistance as they harbor rather diverse resistance genes and possibly promote the distribution of these genes in phylogenetically diverse bacteria (29). In light of the potential health risk, many studies have focused on antibiotic-resistant bacteria recovered from various ecosystems (1, 18, 45). Environments that contain antibiotic residues are particularly worrisome because antibiotics could exert selective pressure and might contribute to the appearance of resistant bacteria. Hospital sewage was once considered the major source of antibiotics in aquatic environments, followed by municipal, agricultural, and aquacultural wastewater (WW), which have also been shown to be important sources of these compounds and resistant bacteria (40). It has also been reported that treated antibiotic production wastewater contains much higher concentrations of antibiotic residues than other aquatic environments (20, 26, 27) and can serve as an important reservoir of resistant bacteria and genes (25).

In the current study, we investigated resistance profiles of bacterial isolates from a unique wastewater treatment plant (WWTP) that is used solely for treating oxytetracycline (OTC), avermectin, and ivermectin production wastewater from the facility of the North China Pharmaceutical Group Corporation in Hebei Province, China. Avermectin and ivermectin are both broad-spectrum antiparasitic agents without any antibacterial or antifungal activities. The effluent of the WWTP is discharged directly into the receiving river without disinfection, and residual levels of OTC in wastewater and downstream sampling points nearly approach those in human blood after drug administration (15, 26). Thus, the characteristics of bacterial strains in heavily OTC-contaminated wastewater and surface water could be unique. In a 1-year (2002-2003) survey of antibiotic resistance in 140 clinical enterococcal isolates from a hospital in this region, the prevalence of tetracycline resistance ranged from 14.9% to 25.0% (47). This rate increased to 47.9 to 75.9% for 302 clinical enterococcal isolates from the same hospital over the following 3 years (2004-2006)

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(28), indicating that tetracycline resistance among human pathogens in this region is becoming more prevalent. Elucidation of the resistance characteristics of bacterial isolates from OTC wastewater and surface water might help explain the prevalence of increased resistance among human pathogens. To gain a comprehensive understanding of this relationship, we isolated bacterial strains from wastewater (WW) and river water downstream (RWD) and river water upstream (RWU) samples using nonselective culture media. Previously, it was shown that long-term administration of a single antibiotic can lead to MDR, a phenomenon that has been observed in bacteria obtained from the urinary tract, intestine, and other niches in both clinical therapy and livestock raising (11, 23, 34). Thus, the combination of long-term treatment and discharge of antibiotic production wastewater might contribute to MDR in environmental bacteria in this specific ecosystem. To test this hypothesis, we determined the resistance profiles of bacterial isolates for 10 antibiotics representing seven classes.

OTC belongs to the tetracycline class of antibiotics, which includes tetracycline, chlortetracycline, doxycycline, minocycline, and glycylcyclines (6). This class of antibiotics represents broad-spectrum agents that act against a range of Gram-positive and Gram-negative bacteria by inhibiting protein synthesis. The tetracyclines have played an important role in human and veterinary medicine, and some have been used as growth promoters for livestock and aquaculture. However, resistance to tetracyclines in many commensal and pathogenic bacteria emerged soon after the widespread application of this class of antibiotic. More than 40 types of tetracycline resistance determinants have been described to date and grouped into three main classes: energy-dependent membrane-associated efflux proteins, which export tetracycline out of the cell; ribosomal protection proteins, which interact with the ribosome and disrupt the tetracycline binding site; and tetracycline inactivation enzymes (6, 37). Many studies of tetracycline resistance (*tet*) genes in various environmental niches have been reported. Resistance genes encoding efflux proteins and ribosomal protection proteins have been identified in phylogenetically diverse bacterial genera. Extensive reviews with detailed information about *tet* genes and their distribution are available (4, 6, 24, 37). Notably lacking, however, is information about *tet* genes in aquatic environments with high levels of antibiotic residue. Studies have shown that several *tet* genes are associated with mobile genetic elements (6, 37) and that the SOS response induced by antibiotics and other factors can promote horizontal dissemination of mobile genetic elements like genomic islands, as well as integron recombination, among bacterial populations (5, 17, 41). In the current study, the presence and distribution of 23 *tet* genes in bacterial isolates from wastewater and river water were determined by PCR. Class I integrons were also examined in the bacterial isolates to evaluate the possibility of horizontal transfer of these resistance genes and/or the promoted dissemination of mobile genetic elements in such heavily antibiotic-polluted environments. We also compared the characteristics of the recovered bacteria with those of isolates from samples from water upstream of the discharge site in the river. Our results reveal the potential environmental influence of wastewater discharge on acquired antibiotic resistance.

MATERIALS AND METHODS

Study site and sampling. Approximately 800 tons of OTC are produced annually by the North China Pharmaceutical Group Corporation facility, located in Hebei Province, China. At this facility, OTC crystal mother liquor (approximately 480 m^3 day^{-1}) is mixed with dilution water (approximately 14,000 m^3 day^{-1}), which consists mainly of discharged circulating cooling water and rinse water from the fermentation tanks and plate-and-frame filter cloth, as well as wastewater (approximately 680 m^3 day^{-1}) from the avermectin and ivermectin production lines. The dilution water undergoes anaerobic treatment before mixing, and the combined waste streams are treated in succession in a sequence batch reactor and a continuous-flow activated sludge reactor. OTC wastewater is the only antibiotic wastewater treated in this plant. The treated waste effluent is discharged directly into the receiving river, the Xiao River (approximately 420,000 m^3 day^{-1}), which is the main source of irrigation water for nearby farmland. Sampling consisted of three sampling campaigns performed over three successive days in December 2004, April 2005, and August 2005. No rain event was registered either during the week previous to or on the sampling days. The WWTP was in operation for about 1 year before the first sampling campaign. Wastewater effluent and receiving river water samples from both downstream and upstream sections were collected in 4-liter brown glass bottles and kept at 4°C in the dark for no more than two days before being analyzed. The upstream and downstream sampling points were approximately 5 km and 20 km from the wastewater discharge point, respectively. Several other wastewater and surface water samples from intermediate points in the wastewater treatment process and different sections of the river were simultaneously obtained to analyze residual OTC concentrations by liquid chromatography-electrospray ionization mass spectrometry. To the best of our knowledge, the wastewater effluent was the only source of antibiotic in the sampled section of this river. Details of the chemical analysis and water characteristics were described previously (26).

Bacterial counts, isolation, and identification. Total cell counts of wastewater effluent and river water samples were estimated by membrane filtration and staining with 4′,6-diamidino-2-phenylindole (DAPI) (43). The isolation and identification of bacterial strains from the water samples has been described (25). Briefly, water samples were first serially diluted and then inoculated onto tryptic soy agar (TSA) and R2A nonselective agar media. After aerobic incubation at 30°C for 24 h, up to 10 colonies with different morphologies were recovered from each plate. After isolates were restreaked three times and purity was verified by microscopy, they were stored at −80°C in tryptic soy broth containing 15% glycerol. Bacterial isolates from the water samples of all three sampling campaigns at a particular sampling site were combined for the following analysis of resistance profiles, *tet* genes, and integrons. CFU were estimated on TSA. The 16S rRNA genes from pure cultures were amplified using bacterial universal primers 27F and 1492R (see Table S1 in the supplemental material) (21). To obtain bacterial DNA for PCR, cultured cells were boiled for 10 min; lysozyme was applied for Gram-positive cells before boiling. One microliter of the boiled bacterial culture was used as the template DNA in standard 50-μl PCR mixtures (Takara, Dalian, China). Amplified products were grouped according to the analysis of HaeIII (Takara, Dalian, China) restriction fragment length polymorphism (RFLP) patterns using BioNumerics version 6.01 (Applied Maths, Sint-Martens-Latem, Belgium). For each RFLP pattern, one or two amplified products were sequenced by Invitrogen, Inc. (Shanghai, China) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems). Bacterial isolates were identified by phylogenetic analysis of 16S rRNA gene sequences with Ribosomal Database Project II release 9.49 and the GenBank database using the BLASTn program (3, 8). Similarity was set above 98% for the species taxonomic level and at 96 to 98% for the genus level.

Antibiotic susceptibility testing. The MICs of antibiotics for each bacterial isolate were determined by a standard 2-fold serial broth microdilution method using Mueller-Hinton broth, according to the Clinical and Laboratory Standards Institute (CLSI) standard guidelines (7), using a concentration range of 0.25 to 1,024 mg $liter^{-1}$. We tested the following 10 antibiotics, representing seven classes (all purchased from Sigma-Aldrich): the tetracyclines OTC, tetracycline (TET), and doxycycline (DOX); the β-lactams ampicillin (AMP) and cefotaxime (CTX); the aminoglycoside kanamycin (KAN); the macrolide erythromycin (ERY); the phenicol chloramphenicol (CHL); the fluoroquinolone ciprofloxacin (CIP); and the ansamycin rifampin (RA). *Escherichia coli* strains ATCC 25922 and ATCC 35218 and *Pseudomonas aeruginosa* strain ATCC 27853 were used as controls. The resistance prevalence for an antibiotic in a bacterial population was calculated as the ratio of the number of strains resistant to the particular antibiotic versus the total number of strains in the population.

Detection of resistance genes and class I integrons. The presence of 23 *tet* genes in all wastewater effluent and downstream river water bacterial isolates was determined by PCR, and class I integrons were checked in all wastewater effluent and river water isolates. Bacterial DNA as template and the standard PCR mixture (50 μl) were the same as described above. The PCR primers and conditions for amplification of *tet* genes and class I integrons are listed in Table S1 in the supplemental material. Amplified products were separated by 2% (wt/vol) agarose gel electrophoresis and visualized by ethidium bromide staining. Bands of interest were purified with a QIAquick PCR cleanup kit (Qiagen, Inc., Chatsworth, CA), and several representative bands were sequenced by Invitrogen Inc. (Shanghai, China) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems). The nearest matches were determined in the GenBank database using the BLASTn program. For all reactions in this study, standard PCR mixtures without DNA template were used as negative controls. Positive bacterial strains carrying *tet* genes or class I integrons verified by sequencing were used as positive controls.

Statistical analysis. The Chao-Jaccard-Est abundance-based similarity index was used to determine similarities in bacterial composition between wastewater effluent and downstream and upstream river water samples using the software program EstimateS, version 8.0 (9). Other statistical analyses, including the Wilcoxon matched-pair test, Mann-Whitney U-test, Kendall's W matched-pair test, and Spearman correlation coefficient, were performed using SPSS, version 16.0 release. The experiment-wise error rate adjustments were performed for multiple comparisons using the Bonferroni adjustment.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of bacterial isolates in this study were deposited in the GenBank database with accession no. FJ950539 to FJ950703, and the nucleotide sequences for several *tet* genes and class I integron genes can be accessed under accession no. FJ950704 to FJ950726.

RESULTS

OTC residue levels. The OTC crystal mother liquor contained extremely high levels of OTC residues (920 ± 20 mg liter^{-1}, average of three sampling campaigns). After mixing with dilution water, the concentration fell to 30.5 ± 1.1 mg liter^{-1} (average of three sampling campaigns). OTC was only partially removed during the wastewater treatment processes, and relatively high levels of OTC residue (19.5 ± 2.9 mg liter^{-1}, average of three sampling campaigns) remained in the wastewater effluent. The OTC concentration in the receiving river decreased from 641 ± 118 μg liter^{-1} (average of three sampling campaigns) at the discharge point to 377 ± 142 μg liter^{-1} (average of three sampling campaigns) at the last sampling site, a distance of approximately 20 km from the discharge point. No significant variations in OTC residue levels in the wastewater samples were observed among the three sampling campaigns. The OTC concentrations in the downstream water samples were lower in August (235 ± 55 μg liter^{-1}, three-day average at the last sampling point) than in December (484 ± 50 μg liter^{-1}, three-day average at the last sampling point) and April (411 ± 97 μg liter^{-1}, three-day average at the last sampling point), possibly due to higher temperatures, stronger sun illumination, and greater surface water flow in the summer. In all upstream water samples, OTC was undetectable (limit of detection, 1 μg liter^{-1}).

Total bacterial counts. The total bacterial cell counts in wastewater (WW), river water downstream (RWD), and river water upstream (RWU) were approximately (3.3 ± 1.9) × 10^7, (2.3 ± 1.5) × 10^6, and (1.7 ± 0.7) × 10^6 cells/ml (average of three sampling times), respectively; the corresponding CFU on TSA were (4.5 ± 2.1) × 10^4, (1.4 ± 0.9) × 10^3, and (2.1 ± 1.1) × 10^3 CFU/ml (average of three sampling times), respectively. The ratio of CFU versus total cell count ranged from 0.06% to 0.14%.

Compositions of bacterial communities. In total, 189, 87, and 65 bacterial isolates were recovered and identified from WW, RWD, and RWU, respectively. As shown in Table 1, most of these isolates were identified at the species level by phylogenetic analysis of bacterial 16S rRNA gene sequences, with the remainder identified at the genus level. Almost all the bacterial species or genera were common residents of wastewater or freshwater environments. Two *Escherichia coli* strains were recovered from WW, which was possibly due to the presence in the industrial wastewater of a very small volume of domestic wastewater from the workers of this facility. Most of the isolates from the three sampling sites belonged to the class *Gammaproteobacteria*, probably reflecting the preferential growth of this bacterial group on the TSA and R2A media used in this study. The other bacterial isolates were affiliated mainly with the *Alphaproteobacteria*, *Betaproteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. The most abundant bacterial group in WW, representing more than half of the isolates, was *Pseudomonas putida* isolates, followed by *Stenotrophomonas maltophilia* isolates. In RWD and RWU, several *Pseudomonas* spp., including *P. fluorescens*, *P. fragi*, and *P. putida*, comprised the majority of isolates. A total of 31, 24, and 15 bacterial species or genera were identified in WW, RWD, and RWU, respectively. Nine species were common to the bacterial communities of WW and RWD, five species were common to RWU and RWD, and three species were common to WW and RWU. Based on Chao-Jaccard-Est abundance analysis, the similarity was 0.536 between the bacterial communities of RWD and WW and 0.591 between RWD and RWU.

Antibiotic resistance prevalence and levels. The resistance prevalence for almost all antibiotics tested in this study was high in WW and RWD isolates, with the exception of the fluoroquinolone CIP (Table 2). There was no significant difference in resistance prevalence between WW and RWD isolates for all antibiotics (Wilcoxon matched-pair test, $P = 0.76$). The levels of resistance prevalence in both WW and RWD isolates were significantly higher than in RWU isolates (Wilcoxon matched-pair test, both P values were <0.01). Although the WWTP that is the focus of the current study is used solely for treating OTC production wastewater, none of the bacterial communities of the WW or RWD exhibited significantly higher resistance prevalence for the tetracyclines, including OTC, TET, and DOX, than for the other classes of antibiotics (Mann-Whitney U-test; both P values were >0.08). The resistance prevalence for DOX was consistently the lowest (1.5% to 83.1%) among the three tetracyclines (1.5% to 95.2%) in all three water samples; the resistance prevalence was lower for CTX (1.5% to 71.4%) than for AMP (24.6% to 93.1%), both of which are β-lactams.

Most bacterial species that were of a sufficient sample size (5 or more strains) in WW exhibited similar resistance characteristics. Only strains of *Ochrobactrum* species exhibited close to significantly lower resistance prevalence than *Pseudomonas* species, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, and *Sphingobacterium* species strains (Wilcoxon matched-pair test, all P values were <0.008). In RWD, only the isolates of *Stenotrophomonas maltophilia* showed close to significantly higher resistance prevalence than *Pseudomonas fragi*, *Pseudo-*

TABLE 1. Affiliations of bacterial strains isolated from wastewater effluent and downstream and upstream river water[a]

Genus or species	No. of isolates from[b]:			Total no. of isolates
	WW	RWD	RWU	
Achromobacter sp.	3			3
Achromobacter xylosoxidans	1			1
Acinetobacter haemolyticus	1			1
Agromyces mediolanus	2			2
Arthrobacter oxydans			1	1
Arthrobacter protophormiae	3			3
Arthrobacter sp.	3			3
Bacillus aquimaris	1			1
Bacillus cereus		5	1	6
Bacillus endophyticus			1	1
Bacillus firmus	2			2
Bacillus pumilus		1		1
Bacillus thuringiensis			1	1
Bacillus sp.	6	1		7
Brevundimonas aurantiaca			1	1
Brevundimonas diminuta	3			3
Caryophanon sp.	2			2
Comamonas testosteroni	1			1
Corynebacterium coyleae			1	1
Corynebacterium sp.			2	2
Enterobacter ludwigii		1		1
Enterobacter sp.		1		1
Escherichia coli	2	1		3
Exiguobacterium aurantiacum		1		1
Flavobacterium denitrificans	1			1
Klebsiella sp.		2		2
Microbacterium oxydans	3	1		4
Microbacterium sp.	1			1
Morganella morganii		1		1
Ochrobactrum grignonense	5	1		6
Ochrobactrum sp.	7			7
Paracoccus versutus	1			1
Pseudochrobactrum asaccharolyticum	4			4
Pseudochrobactrum kiredjianae	1			1
Pseudochrobactrum saccharolyticum	1			1
Pseudochrobactrum sp.	1	2		3
Pseudomonas fluorescens	4	14	39	57
Pseudomonas fragi		16	2	18
Pseudomonas grimontii		1		1
Pseudomonas mandelii			1	1
Pseudomonas monteilii		1		1
Pseudomonas plecoglossicida		1		1
Pseudomonas psychrophila		4		4
Pseudomonas putida	75	14	8	97
Pseudomonas resinovorans	1			1
Pseudomonas veronii		2		2
Pseudomonas sp.	19	7	4	30
Rhodococcus erythropolis	1			1
Sphingobacterium faecium	2			2
Sphingobacterium sp.	5			5
Staphylococcus equorum			1	1
Staphylococcus saprophyticus			1	1
Staphylococcus sp.			1	1
Stenotrophomonas maltophilia	27	7		34
Stenotrophomonas sp.		1		1
Trichococcus flocculiformis		1		1
Total	189	87	65	341

[a] For each sampling site, bacterial isolates from water samples of three sampling times in December 2004, April 2005, and August 2005 were combined.
[b] WW, wastewater effluent; RWD, downstream river water; RWU, upstream river water.

monas fluorescens, *Bacillus cereus*, and *Pseudomonas putida* (Wilcoxon matched-pair test, all P values were <0.009). The resistance characteristics of most bacterial species with a sufficient sample size in RWD were also similar. No significant difference was observed between the resistance prevalence of *Pseudomonas fluorescens* and *Pseudomonas putida* in RWU (Wilcoxon matched-pair test, $P = 0.37$).

As shown in Table 2, the antibiotic resistance levels of the bacterial communities in all three water samples were reflected by the MIC_{50}s and MIC_{90}s, which represent MICs required for the inhibition of 50% and 90% of bacterial strains, respectively. No significant differences in the MIC_{50} or MIC_{90} values of all 10 antibiotics were observed between the bacterial communities of WW and RWD (Wilcoxon matched-pair test, both P values were >0.1). Both communities exhibited significantly higher MIC_{50} and MIC_{90} values than the RWU isolates (Wilcoxon matched-pair test, all P values were <0.01). Similar to the results for antibiotic resistance prevalence, the MIC_{50} and MIC_{90} values of the three tetracyclines were not significantly higher than those of the other classes of antibiotics in all three water samples (Mann-Whitney U-test, all $P > 0.07$), and the MIC_{50} and MIC_{90} values of DOX were always the lowest among the three tetracyclines. Those of CTX were also lower than those of AMP. The differences in antibiotic resistance levels of particular bacterial species or genera from the three water samples were not significant.

It also needs to be noted that the majority of bacterial isolates (86.8%) from WW exhibited MIC values for OTC that were higher than the residual levels of OTC in the wastewater effluent (19.5 ± 2.9 mg liter^{-1}, average of three sampling campaigns). Meanwhile, 96.6% of bacterial isolates from RWD exhibited MIC values for OTC that were higher than the OTC levels at the corresponding river sampling site (377 ± 142 μg liter^{-1}, average of three sampling campaigns), and 81.6% of RWD isolates exhibited MIC values for OTC that were higher than the OTC levels in the wastewater effluent. Only 1.5% and 40% of RWU isolates exhibited MIC values for OTC that were higher than the OTC levels in the wastewater effluent and river sampling site, respectively. These results suggested that OTC residues might lead to much higher resistance levels in many environmental bacteria.

MDR. The distribution of the number of antibiotic classes to which bacterial isolates were simultaneously resistant (coresistant) in three water samples is summarized in Fig. 1. Almost all of the WW and RWD isolates (more than 96%) exhibited MDR. The mean numbers of the coresistant antibiotic classes were 5.0 and 5.2 (range, 0 to 7) for the bacterial communities of WW and RWD, respectively. These values were significantly higher than that of the RWU isolates (mean, 1.0; range, 0 to 3). Approximately 27.7% of the RWU isolates exhibited MDR, and the majority of these MDR isolates (77.8%) were coresistant to two classes of antibiotics. When the bacterial isolates of the three water samples were pooled, antibiotics within the same class correlated more closely with each other than with unrelated antibiotics. For example, for the tetracyclines OTC, TET and DOX, the Spearman correlation coefficients ranged from 0.738 to 0.985, and for the β-lactams AMP and CTX, the Spearman correlation coefficient was 0.601. The Spearman correlation coefficients between unrelated antibiotics were generally lower than 0.6.

TABLE 2. Activities of 10 antibiotics against isolates in wastewater effluent and downstream and upstream river water[a]

Antibiotic	Activity[c] against isolates from[b]: WW				RWD				RWU			
	Resistance prevalence (%)	MIC (mg liter^{-1}) Range	50%	90%	Resistance prevalence (%)	MIC (mg liter^{-1}) Range	50%	90%	Resistance prevalence (%)	MIC (mg liter^{-1}) Range	50%	90%
Oxytetracycline	94.7	1 to >1,024	512	>1,024	86.2	0.5 to >1,024	128	1,024	3.1	0.25 to 128	0.5	2
Tetracycline	95.2	1 to >1,024	512	>1,024	85.1	1 to >1,024	256	1,024	3.1	0.25 to 128	0.5	2
Doxycycline	83.1	0.5 to 1,024	128	256	70.1	0.5 to 1,024	64	512	1.5	0.25 to 64	0.5	1
Ampicillin	85.2	0.5 to >1,024	256	1,024	93.1	1 to >1,024	256	512	24.6	0.25 to 1,024	8	128
Cefotaxime	71.4	0.25 to 1,024	64	256	59.8	0.5 to 1,024	128	512	1.5	0.25 to 256	0.25	1
Kanamycin	55.0	0.25 to >1,024	128	512	70.1	0.5 to >1,024	256	1,024	4.6	0.25 to 128	1	8
Chloramphenicol	72.5	0.5 to 1,024	128	512	82.8	0.5 to 1,024	128	512	9.2	0.25 to 512	2	16
Ciprofloxacin	9.0	0.25 to 128	0.5	4	6.9	0.25 to 32	0.25	4	7.7	0.25 to 32	0.25	0.25
Erythromycin	92.1	0.25 to >1,024	128	1,024	94.3	0.5 to 1,024	128	512	24.6	0.25 to 512	4	64
Rifampin	88.4	0.5 to >1,024	128	1,024	92.0	0.25 to 1,024	128	512	26.2	0.25 to 512	2	128

[a] The *Exiguobacterium* spp. and *Microbacterium* spp. strains for which there were still no interpretive criteria according to CLSI standards were not taken into account when determining antibiotic activities.
[b] WW, wastewater effluent; RWD, downstream river water; RWU, upstream river water.
[c] Resistance prevalence for the indicated antibiotic in the bacterial population was calculated as the ratio of the number of resistant strains versus the total number of strains in the population. 50%, MIC_{50}; 90%, MIC_{90}. The range of MICs of each antibiotic for all tested isolates in WW, RWD, and RWU, respectively, is shown.

Tetracycline resistance genes. Most of the WW and RWD isolates (prevalence of 94.2% and 95.4%, respectively) harbored *tet* genes. These *tet*-positive isolates were generally not susceptible to tetracyclines (MICs greater than 4 mg liter^{-1}). As shown in Table 3, in WW isolates, *tet* genes encoding efflux proteins were clearly more abundant than those encoding ribosomal protection proteins. The most common resistance gene was *tet*(A), identified in 69.3% of the WW isolates, followed by *tet*(C), *tet*(L), and *tet*(J) (in 42.9%, 37.6%, and 32.3% of the isolates, respectively). All of these resistance genes encode efflux proteins. The most common ribosomal protection protein determinant was *tet*(W), found in 28.0% of all WW isolates, followed by *otr*(A) and *tet*(M) (in 16.9% and 12.2%, respectively). Resistance genes encoding ribosomal protection proteins were generally more abundant in RWD isolates. *tet*(W) was the most abundant, found in 67.8% of the RWD isolates, followed by *tet*(S), *tet*(M), and *tet*(O) in 33.3%, 18.4%, and 16.0% of the isolates. For genes encoding efflux proteins, *tet*(A) was the most common, detected in 62.1% of the RWD isolates, followed by *tet*(J), *tet*(D), and *tet*(L) (in 31.0% to 44.8% of the isolates). Above all, there was no significant

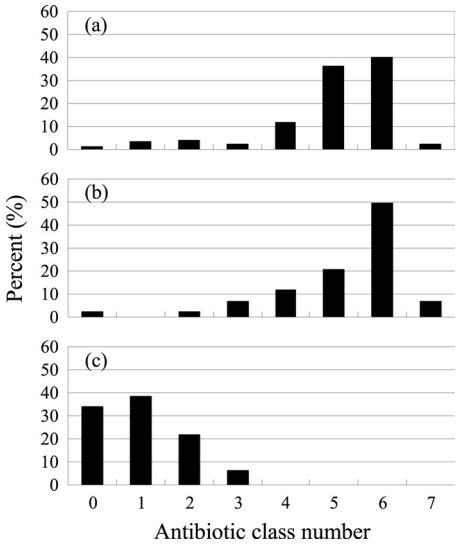

FIG. 1. Multidrug resistance in the wastewater effluent (a), downstream river water sample (b), and upstream river water sample (c). The *x* axis represents the number of antibiotic classes to which an isolate was simultaneously resistant, and the *y* axis represents the percentage of that resistance profile in the bacterial population.

TABLE 3. Prevalence of bacterial isolates carrying *tet* genes in bacterial populations in wastewater and river water downstream

Mechanism of drug resistance[b]	Resistance gene	Prevalence of isolates (%) in[a]: WW	RWD
Efflux	*tet*(A)	69.3	62.1
	tet(B)	6.3	6.9
	tet(C)	42.9	21.8
	tet(D)	26.5	33.3
	tet(E)	2.1	0
	tet(G)	0	0
	tet(H)	0.5	1.1
	tet(J)	32.3	44.8
	tet(Z)	23.3	9.2
	tet(30)	2.6	9.2
	tet(K)	23.3	16.1
	tet(L)	37.6	31.0
	tet(AP)	11.6	12.6
	tet(Y)	22.2	27.6
Ribosomal protection	*tet*(M)	12.2	18.4
	tet(O)	9.0	16.1
	tet(S)	6.3	33.3
	tet(W)	28.0	67.8
	tet(Q)	0	0
	tet(T)	4.2	5.7
	otr(A)	16.9	8.0
	tet(BP)	0.5	0
Enzymatic modification	*tet*(X)	0	0

[a] WW, wastewater effluent; RWD, downstream river water.
[b] Efflux is the mechanism of utilizing energy-dependent membrane-associated efflux proteins which export tetracycline out of the cell; ribosomal protection is the mechanism of utilizing ribosomal protection proteins which interact with the ribosome and disrupt the tetracycline binding site; and enzymatic modification is the mechanism of utilizing tetracycline inactivation enzymes.

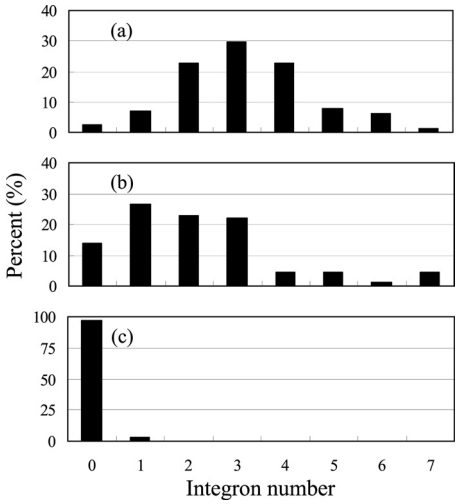

FIG. 2. The number of integrons carried by isolates in the wastewater effluent (a), downstream river water sample (b), and upstream river water sample (c). The *x* axis represents the number of integrons carried by an isolate, and the *y* axis represents the percentage of that integron profile in the bacterial population.

difference in the prevalence of all *tet* genes between WW and RWD isolates (Wilcoxon matched-pair test, $P = 0.61$). When all WW and RWD isolates were pooled, *tet*(A) was the most common resistance gene (67.0%), followed by *tet*(W), *tet*(C), *tet*(J), *tet*(L), *tet*(D), *tet*(Y), and *tet*(K) (in 21.0% to 40.6% of the isolates). *tet* genes were carried mainly by *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, *Ochrobactrum*, *Sphingobacterium*, *Pseudochrobactrum*, *Arthrobacter*, *Microbacterium*, *Achromobacter*, *Escherichia*, and *Brevundimonas* species. A detailed list of bacterial species or genera carrying *tet* genes and the identities of the genes is presented in Table S2 in the supplemental material.

Bacterial isolates that carried more than one kind of *tet* gene were common in WW and RWD. Among WW and RWD isolates, 86.2% and 87.4%, respectively, harbored two or more *tet* genes. Some strains of *Pseudomonas putida*, *P. fragi*, *Escherichia coli*, and *Stenotrophomonas maltophilia* carried seven to nine *tet* genes (the detailed combinations of *tet* genes are not shown). The mean number of *tet* genes carried by a single isolate was 3.8 and 4.3 for the bacterial communities of WW and RWD, respectively. Previous studies have indicated that the number of *tet* genes carried by one bacterial strain was not related to the MIC for this strain (42). In our study, the numbers of *tet* genes and the MIC values of the three tetracyclines correlated positively (Spearman correlation coefficient, all *P* values were <0.001, $n = 276$), although the coefficients were limited (ranging from 0.264 to 0.275). Furthermore, there was some degree of correlation between DOX resistance and carriage of *tet*(C) and *tet*(A) (Spearman correlation coefficients of 0.206 and 0.195, all *P* values were <0.001, $n = 276$).

Class I integrons. Class I integrons were identified in 97.4% of WW isolates and 86.2% of RWD isolates (Fig. 2). The number of integrons carried by the WW isolates was significantly higher than the number carried by the RWD isolates (Mann-Whitney U-test, $P = 0.000$, means of 3.2 and 2.2, respectively). Some *Pseudomonas* sp. and *Bacillus* sp. isolates from WW and RWD contained seven different integrons simultaneously, based on the lengths of amplified PCR products. Only two RWU isolates contained integrons. Although class I integrons in WW and RWD isolates were relatively abundant, the antibiotic resistance genes in the gene cassettes of these integrons were not highly diverse. Only three groups of resistance genes were found in the sequenced integrons: aminoglycoside resistance genes (*aadA1*, *aadA2*, and *aadA2a*), which encode aminoglycoside adenyltransferases that confer KAN, streptomycin, and spectinomycin resistance; trimethoprim resistance genes (*dfrA1* and *dfrA12*), which encode dihydrofolate reductase; and the quaternary ammonium compound resistance gene *qacG*. Aminoglycoside and trimethoprim resistance genes were distributed widely in the isolates; however, no significant correlation between the presence of *aadA* and the KAN resistance phenotype was observed for all the isolates of this study. The quaternary ammonium compound resistance gene *qacG* was identified in only one WW isolate.

DISCUSSION

The isolates of this study were mainly grouped into the class *Gammaproteobacteria*, which should be due to the favored growth of this bacterial group in nutrient-rich culture media. The results of clone libraries of bacterial 16S rRNA partial genes for the same water samples used in this study indicated that the classes *Betaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*, as well as the phyla *Bacteroidetes* and *Firmicutes*, were generally dominant bacterial groups (details not shown). The bias with culture-based analysis might not be a serious problem, since the same culture techniques were used for WW, RWD, and RWU, allowing for valid comparison of the resistance prevalence, levels, and genes among the different locations.

The antibiotic resistance prevalence and levels in WW and RWD isolates were generally much higher than those reported previously for municipal wastewater, hospital wastewater, and surface water (16, 19, 36). Such high resistance levels have only been recorded in some aquaculture studies, where OTC or other antibiotics, such as sulfonamides and chloramphenicol, were used as supplements in fish feed or poured directly into the water. Antibiotic residues can persist in fish farm sediments for a long time, resulting in MICs of 64 to 2,048 mg liter^{-1} and MIC_{90}s of 1,024 to 2,048 mg liter^{-1} for OTC-resistant bacteria (33). Previously, we investigated the bacterial characteristics of a penicillin production wastewater treatment plant and the receiving river and demonstrated that high resistance prevalence and levels could be induced by long-term penicillin exposure (25). However, perhaps due to the much lower levels of penicillin residue (levels of 1.68 ± 0.48 μg/liter in treated wastewater effluent and from 0.31 ± 0.04 μg/liter to under the detection limit in the receiving river) compared to the levels of OTC in the current study, there were some clear differences between the characteristics of the bacterial communities in these two ecosystems. First, the resistance prevalence and levels for β-lactams, including penicillin, were significantly higher than for the other classes of antibiotics examined in the penicillin ecosystem in both the wastewater and downstream river. However, in the current study, no significant differences in resistance prevalence of the three tetracyclines and the other

classes of antibiotics examined were observed in WW or RWD. Second, the resistance prevalence and levels of all antibiotics in treated penicillin production wastewater were significantly higher than in the penicillin-receiving river. In the OTC ecosystem, there was no significant difference in resistance characteristics between WW and RWD. These results suggest that the resistance prevalence and levels of unrelated antibiotics in a bacterial community may increase along with the concentration of the antibiotic under study in the aquatic environment and that discrepancies of resistance prevalence and levels between the target antibiotic and unrelated antibiotics might ultimately become negligible. Furthermore, despite significant differences in OTC levels between WW and RWD samples, the antibiotic resistance prevalence and levels were similar between the two communities, suggesting that resistance characteristics may not be affected by antibiotic concentrations above several hundred $\mu g\ liter^{-1}$ (i.e., the OTC levels in RWD of this study). Although numerous environmental studies of antibiotic-resistant bacteria have been reported to date, corresponding information about antibiotic residue concentrations, especially when the levels are high, is still scarce. The relationship between antibiotic concentration and the resistance characteristics of bacterial communities needs to be clarified by additional studies. It was interesting that the resistance prevalence and levels for DOX were lower than for OTC and TET in the water sample isolates; a similar phenomenon was observed for CTX compared to AMP. These results might be due to the fact that DOX and CTX are relatively new antibiotics with higher activities.

Several studies have clearly demonstrated that the administration of even a single antibiotic can select for MDR strains during human or veterinary clinical therapy (11, 23, 34). In the current study, MDR was observed in most WW and RWD isolates, possibly due to long-term exposure of the microorganisms to high concentrations of OTC. It has been suggested that MDR, caused by long-term selection pressure of a single antibiotic, is linked to mobile genetic elements, such as transposons and plasmids harboring diverse resistance genes (22, 23). The dissemination of these elements in bacterial populations has also been shown to be promoted by antibiotic selection pressure (5). Integrons are natural gene expression systems that can capture promoterless gene cassettes through site-specific recombination and transfer between bacterial strains by associating with other mobile gene elements, like conjugative plasmids and transposons. Integrons, especially class I integrons, commonly contain antibiotic resistance gene cassettes and are closely related to MDR, generally by containing several resistance gene cassettes simultaneously (29). In this study, the presence of class I integrons was investigated in all isolates. Integrons were indeed distributed universally in WW and RWD isolates, providing evidence that residual OTC may promote the transmission of mobile genetic elements. However, the diversity of antibiotic resistance genes harbored by these integrons was low, and many contained only short sequences without any open reading frame (data not shown). No *tet* genes were detected in any gene cassette of an integron, and there was no significant correlation between the presence of *aad* in the integron and KAN resistance of the isolate, in spite of the widespread carriage of *aad* among the isolates. These results indicated that class I integrons may play a relatively unimportant role in the MDR found in this study. Considering that integrons are generally much smaller gene elements than plasmids or transposons, we speculate that plasmids or transposons might be the main mechanism of MDR. More studies focusing on antibiotic determinants carried by plasmids and transposons are needed in the future. A multidrug efflux pump could also be contributing to MDR in this ecosystem, as shown previously for many pathogens (35).

Several bacterial genera that have not previously been described as harboring *tet* genes were identified in the current study, such as *Pseudochrobactrum*, *Caryophanon*, *Trichococcus*, *Agromyces*, and *Exiguobacterium* species. The distribution of *tet* genes in this study was generally in agreement with earlier reports (6, 37; see also http://faculty.washington.edu/marilynr/). The most common *tet* genes in WW and RWD were *tet*(A), *tet*(W), *tet*(C), *tet*(J), *tet*(L), *tet*(D), *tet*(Y), and *tet*(K), the majority of which are frequently referred to in the literature (37). The distribution of *tet*(B) and *tet*(M), which have been reported to be widely spread among diverse bacterial hosts, was relatively limited in either the number of positive bacterial genera or species or the prevalence of positive isolates among WW and RWD in this study. To date, *tet*(J) and *tet*(Y) have been individually identified in only three bacterial species (http://faculty.washington.edu/marilynr/). In this study, we detected these genes in several bacterial genera or species that have previously been reported as positive, including *Escherichia coli* and *Morganella* spp., as well as for the first time in other species, such as *Achromobacter* species, *Brevundimonas diminuta*, *Enterobacter*, *Ochrobactrum*, *Pseudochrobactrum*, *Pseudomonas*, and *Sphingobacterium* species, and *Stenotrophomonas maltophilia*. *tet*(A) and *tet*(W), encoding efflux proteins and ribosomal protection proteins, respectively, were the most abundant resistance genes in this study. With increasing screening, *tet*(A) and *tet*(W) have been identified in more and more bacterial genera or species. The association of these genes with plasmids and conjugative transposons may underlie their wide distribution in the current and previous studies (31, 44). Many *tet* genes have been linked to mobile genetic elements like integrons, plasmids, or transposons (2, 6, 37). The number of *tet*-positive bacterial genera or species and the prevalence of *tet*-positive WW and RWD isolates were in general higher in the current study than in previous studies (32, 38, 39). This phenomenon could be due to the high concentration of residual OTC in the current ecosystem, which promotes horizontal transfer of mobile genetic elements, such as plasmids or transposons, along with the associated *tet* genes.

In summary, the bacterial isolates from a heavily OTC-polluted aquatic environment exhibited unique characteristics, both in phenotype and genotype. Although no pathogens were isolated in this study, the high prevalence of indigenous antibiotic-resistant bacteria harboring diverse resistance genes could represent a potential health risk. Many pathways exist by which environmental bacteria can be acquired by humans, such as swallowing during swimming in surface water and consumption of raw vegetables irrigated with contaminated water. Antibiotic resistance genes might be then transferred to the human microbiota, including pathogens, particularly under antibiotic pressures, as well as via the SOS response (5, 17, 41).

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