Bacterial community characteristics under long-term antibiotic selection pressures

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ABSTRACT

To investigate bacterial community characteristics under long-term antibiotic selection pressures, water samples from the upstream and the downstream sections of two rivers individually receiving the treated penicillin G and oxytetracycline production wastewater, as well as the anaerobic and the aerobic effluent of the penicillin G production wastewater treatment plant, were taken and analyzed. Antibiotic resistance ratios of bacterial communities in water samples were estimated by culture-based analysis. The majority of bacterial colonies (approximately 55%–70%) in both downstream rivers and the aerobic effluent showed resistance to 80 µg/ml of antibiotics tested, while the resistance ratios were less than 10% and 5% respectively for both upstream rivers. Six 16S rRNA gene clone libraries were constructed with 355 sequences and 215 OTUs totally obtained representing 465 clones. The antibiotic stresses seemed not reduce the diversities of bacterial communities in antibiotic containing water samples compared to those in the two reference upstream rivers. Bacterial groups present in the two reference upstream rivers were common residents in freshwater ecosystems, with the dominant groups as the phyla *Proteobacteria* including *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, as well as *Actinobacteria* and *Bacteroidetes*. The phyla *Proteobacteria* and *Firmicutes* were dominant in all antibiotic containing water samples, with the clones belonged to *Deltaproteobacteria* and *Epsilonproteobacteria* significantly abundant, as well as Gram-positive low GC bacteria in the classes *Clostridia* and *Bacilli*. It thus seemed that *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* might be specifically associated with antibiotic containing environments.

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1. Introduction

Environments containing high concentrations of antibiotics are normally anthropogenic, such as municipal, hospital, stock raising and pharmaceutical producing wastewater, as well as polluted surface water and fishery ponds. Antibiotics in these aquatic environments could directly act on bacterial strains and exert selective pressure, resulting in the change of

bacterial community structure inevitably. However, little is known about the taxonomic composition of the whole bacterial community in antibiotic polluted environments. Although numerous researches have been performed to investigate antibiotic resistance characteristics of bacterial isolates from different environmental sources including stock farms, poultry farms, fisheries, surface water, and lakes (Wittwer et al., 2005; Huddleston et al., 2006; Jindal et al., 2006;

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Hamelin et al., 2007), most of these researches were focused on human related pathogens (Huddleston et al., 2006; Hamelin et al., 2007), and the remaining generally isolated environmental bacteria using non-selective rich nutrient media at aerobic conditions (Miranda and Zemelman, 2002; Messi et al., 2005). *Gammaproteobacteria* like *Pseudomonas* spp. was generally the dominant bacterial group in these culture-based studies, mainly due to the favored growth of *Gammaproteobacteria* in nutrient rich culture media. Several other bacterial groups such as *Bacteroidetes*, *Actinobacteria*, *Betaproteobacteria* and *Alphaproteobacteria* were also recovered with comparably lower colony numbers. As the majority of bacterial species in environments still could not be isolated and cultured, the above culture-based researches could not reflect the actual composition of the whole community. Some particular bacterial groups which are difficult to be cultured at normal conditions might be dominant in environments containing antibiotics.

Furthermore, under the selection pressure of antibiotics, most of bacterial strains might become resistant to antibiotics. Some metagenomic researches have demonstrated that the diversity of antibiotic resistance genes in environments is greater than previously accounted for basing on cultured bacteria, indicating that many resistance genes are actually carried by uncultured bacteria (Riesenfeld et al., 2004; D'Costa et al., 2006; Sommer et al., 2009). The whole bacterial community including both cultured and uncultured bacteria constitutes an important reservoir of antibiotic resistance genes which could furthermore move into pathogens via horizontal gene transfer facilitated with mobile gene elements such as plasmids, transposons and so on (Thomas and Nielsen, 2005), as confirmed by numerous investigations about antibiotic resistant pathogenic bacteria (Martínez et al., 2007). The elucidation of bacterial community composition in antibiotic containing environments would thus help to suggest the potential antibiotic resistant bacterial groups, which might be important carriers of resistance genes and sometimes the source of clinically important resistance genes (Martínez, 2008).

To our knowledge, few researches have been performed to elucidate the whole bacterial community structures in antibiotic containing environments until now. Córdova-Kreylos and Scow have elucidated the effects of ciprofloxacin on salt marsh sediment microbial communities by using phospholipid fatty acid (PLFA) analysis (Córdova-Kreylos and Scow, 2007). However, their conclusions were acquired through lab-experiment, which might not be able to reflect the situations in actual environments. Thus in this study, two rivers individually receiving the treated penicillin G and oxytetracycline production wastewater, which were discharged from two antibiotic producing facilities and both contained significantly higher concentrations of antibiotics than normal aquatic environments (Li et al., 2008a,b), were selected to investigate bacterial community characteristics under long-term antibiotic stresses. Considering that many environmental factors might influence bacterial community structures, several water samples from the penicillin G production wastewater treatment facility were also obtained and analyzed to compare with river samples. The antibiotic resistance ratios of bacterial communities in water samples

were first estimated using culture-based analysis. Clone libraries of 16S rRNA gene which could provide the detailed and reliable information were then constructed for water samples from each site. The results would help to complement existing knowledge of bacterial community composition in antibiotic containing environments and suggest the possible environmental antibiotic resistant groups still unknown until now.

2. Materials and methods

2.1. Study site and sampling

The two rivers and wastewater treatment plants (WWTPs) were all located in Hebei Province, China. Penicillin G production wastewater is treated in the WWTP which included an anaerobic digestion, a hydrolyzation and two aerobic reactors successively. The treated effluent is discharged into the receiving river, Wangyang River. Meanwhile, the production wastewater from another oxytetracycline producing facility is treated in the WWTP including a sequence batch reactor and a continuous-flow activated sludge reactor, and then discharged into the Xiao River. In December 2004, April and August 2005, surface water samples from upstream (longitude 114°42'13"E and latitude 37°59'8"N for Wangyang River; longitude 114°27'19"E and latitude 38°2'4"N for Xiao River) and downstream sections (longitude 114°53'20"E and latitude 37°52'38"N for Wangyang River; longitude 114°34'39"E and latitude 37°51'50"N for Xiao River) of wastewater discharging points, as well as the effluent samples from the anaerobic and aerobic apartments of the penicillin G production wastewater treatment plant were all taken in 4-L brown glass bottles. Water samples were kept at 4 °C in the darkness for at most two days. Upstream and downstream sampling sites were individually approximately 5 km and 30 km away from the discharging point at the Wangyang River, and 5 km and 20 km away at the Xiao River. Penicillin G and oxytetracycline residues in water samples were all determined using LC-ESI-MS. The detailed analysis methods and the characteristics of water samples could be found elsewhere (Li et al., 2008a,b).

2.2. Culture-based analysis

Water samples from the upstream and downstream sections of the two rivers, as well as the aerobic effluent of the penicillin G production wastewater treatment plant were applied for culture-based analysis. Two sets of plates were simultaneously incubated for each water sample at proper dilution using non-selective Tryptic soy agar (TSA) at 30 °C for 24 h aerobically. Control plate was not added with any antibiotics, then 80 µg/ml ampicillin was added in agar for the upstream and downstream water samples of penicillin G containing river, as well as the aerobic effluent, and 80 µg/ml oxytetracycline was added for the upstream and downstream water samples of oxytetracycline containing river. Ampicillin was used instead of penicillin G due to its broad-spectrum ability. The percentages of the colony numbers of plates added with antibiotics accounting for those of the corresponding control

Table 1 – Distribution of phylogenetic groups among bacterial 16S rRNA gene clone libraries for the upstream and downstream of the two rivers individually receiving treated penicillin G and oxytetracycline production wastewater, as well as the anaerobic and the aerobic effluent of penicillin G production wastewater.

Phylum	Class	No. of OTUs (no. of clones)					
		Upstream river1 ^a	Upstream river2 ^b	Downstream river1 ^a	Downstream river2 ^b	Anaerobic effluent	Aerobic effluent
Proteobacteria	Alpha-	5 (12)	9 (13)	5 (6)	3 (3)	1 (1)	1 (1)
	Beta-	3 (13)	10 (32)	9 (15)	3 (6)	1 (2)	13 (18)
	Gamma-	2 (9)	2 (3)	1 (2)	1 (2)	—	—
	Delta-	—	—	5 (10)	6 (12)	2 (8)	5 (9)
	Epsilon-	—	—	1 (3)	3 (19)	5 (11)	5 (13)
	Unclassified	—	1 (1)	—	1 (1)	—	1 (3)
Firmicutes	Clostridia	1 (1)	—	8 (20)	9 (12)	12 (43)	2 (3)
	Bacilli	1 (5)	—	3 (10)	1 (5)	3 (7)	5 (18)
	Unclassified	—	—	2 (2)	—	1 (4)	1 (2)
Actinobacteria	Actinobacteria	7 (24)	1 (3)	2 (3)	1 (2)	—	—
Planctomycetes	Planctomycetacia	1 (3)	2 (3)	—	—	—	2 (2)
Acidobacteria	Acidobacteria	1 (6)	3 (3)	2 (2)	—	—	—
Bacteroidetes	Bacteroidetes	—	1 (1)	1 (1)	3 (6)	—	2 (3)
	Flavobacteria	—	2 (5)	1 (2)	—	—	—
	Sphingobacteria	1 (1)	5 (5)	1 (1)	—	—	1 (1)
	Unclassified	—	1 (1)	1 (2)	3 (3)	—	1 (3)
Chloroflexi	Anaerolineae	—	—	—	—	—	1 (1)
	Unclassified	—	—	—	—	—	1 (1)
Gemmatimonadetes	Gemmatimonadetes	—	—	1 (1)	—	—	—
Verrucomicrobia	Verrucomicrobiae	—	1 (1)	—	1 (1)	—	—
Lentisphaerae	Lentisphaerae	—	—	—	1 (1)	—	—
Unclassified		1 (1)	1 (2)	2 (2)	3 (4)	1 (1)	3 (3)
Total no.		23 (75)	39 (73)	45 (82)	39 (77)	26 (77)	43 (81)

a The river1 received treated penicillin G production wastewater.

b The river2 received treated oxytetracycline production wastewater.

Fig. 1 – Multiple correspondence analysis of bacterial groups and water samples including upstream and downstream of the two rivers 1 and 2 individually receiving treated penicillin G and oxytetracycline production wastewater, as well as the anaerobic and the aerobic effluent of penicillin G production wastewater. Only bacterial groups with sufficient sample sizes were shown.

plates were used to reflect antibiotic resistance ratios of bacterial community in water samples.

2.3. DNA extraction, PCR, cloning and sequencing of 16S rRNA genes

500-ml water sample was vacuum filtered through a 0.2- μ m-pore-size polyethersulfone membrane filter. Then samples were washed by 10 ml of phosphate buffer (pH 8.0), and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described previously (Crump et al., 1999). The yield and quality of DNA was estimated visually after electrophoresis on 1% (wt/vol) agarose gel through comparison with a molecular mass ladder.

The 16S rRNA gene was amplified using bacteria universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-TACGGYTACCTTGTACGACTT) (Lane, 1991). The standard 50 μ l PCR mixture (Takara, Dalian, China) included 1 $\times$ PCR buffer containing 1.5 mM $MgCl_2$, 200 μ M of each deoxynucleoside triphosphate, 10 pmol of each primer, 1.25 U of TaKaRa rTaq polymerase, and approximately 50 ng of template DNA. PCR conditions were as follows: 95 $^{\circ}$ C for 10 min, followed by 30 cycles of 95 $^{\circ}$ C for 1 min, 55 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 1 min 30 s, and a final extension at 72 $^{\circ}$ C for 15 min. After confirmed by electrophoresis in 1.2% (wt/vol) agarose gel, amplification products were purified with the Qiaquick PCR cleanup kit (Qiagen, Inc., Chatsworth, Calif.). In order to minimize PCR bias in subsequent cloning steps, three separate reactions were run for each sample and pooled together, PCR products of the samples from the same sampling site was also pooled together.

The amplified 16S rRNA gene products were further cloned into the TOPO TA cloning vector pCR2.1, and TOP10 *E. coli* transformants were further selected according to manufacturer's instructions (Invitrogen). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F and M13R under the same conditions with above. PCR products were digested (3 h, 37 $^{\circ}$ C) with HaeIII (Takara, Dalian, China) and separated by electrophoresis through 2% agarose gels. Clones were grouped based on RFLP patterns, and representative clones were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

2.4. Phylogenetic and statistical analysis

DNA sequences were edited manually using BioEdit (Hall, 1999), and then searched against RDP II and the GenBank database (Altschul et al., 1997; Cole et al., 2007). The most similar reference sequences were retrieved and aligned with clone sequences using ClustalX (Thompson et al., 1997). Phylogenetic trees were constructed using MEGA, version 3.1 by the neighbor-joining algorithm and the Jukes-Cantor

distance estimation method (Kumar et al., 2004). Possible chimeras were checked using CHIMERA_CHECK in RDP II.

The sequences sharing 97% or greater similarity were grouped into the same operational taxonomic unit (OTU) and the OTU number was determined using DOTUR (Schloss and Handelsman, 2005). OTU richness S_{Chao1} and S_{ACE} as well as Shannon diversity (H') were calculated using EstimateS version 8.0 with 100 random sample repetitions (Colwell, 2005). Evenness (E') indices were calculated as follows: $E' = H'/\ln n$, where n is OTU number. Coverage (C') was calculated as follows: $C' = 1 - (n_1/N)$, where n_1 is the number of OTUs that occurred once and N is the total number of clones. Rarefaction curves were constructed using aRarefactWin (<http://www.uga.edu/~strata/software.html>). UniFrac computational analysis was performed to compare clone libraries from different sampling sites (Lozupone and Knight, 2005). Clone libraries from different sites were clustered by the application of the UPGMA method to the UniFrac metric matrix, and principal coordinate analysis (PCoA) was also performed with UniFrac metric matrix. Multiple correspondence analysis of specific bacterial groups with a sufficient sample size (5 or more clones) and water samples was also performed by using the SPSS version 16.0 release.

2.5. Nucleotide sequence accession numbers

The 16S rRNA gene sequences could be accessed in the GenBank database under the accession numbers EU234086 to EU234324, EU864431 to EU864494 and FJ230892-FJ230941.

3. Results

3.1. Antibiotics and resistance ratios in water samples

The concentrations of penicillin G decreased from 72.6 ± 3.7 μ g/L in the anaerobic effluent to 1.68 ± 0.48 μ g/L in the aerobic effluent of the WWTP, and ranged from 0.35 μ g/L to under the detection limit (0.031 μ g/L) in the receiving river water samples (Li et al., 2008b). Meanwhile, oxytetracycline was determined in the downstream of Xiao River at 376.7 ± 141.7 μ g/L (Li et al., 2008a). No penicillin G or oxytetracycline could be detected in the reference upstream rivers. The antibiotic residual levels in this study were comparably much higher than those reported in normal aquatic environments previously (Kolpin et al., 2002).

Antibiotic resistance ratios were roughly estimated for all the river water and the aerobic effluent samples using culture-based resistance assay. Approximately 65% of colonies from penicillin G containing downstream water and 70% of the aerobic effluent colonies showed resistance to 80 μ g/ml of ampicillin, with the resistance percentages less than 10% for the reference upstream river. Then approximately 55% of colonies from the oxytetracycline containing downstream

Fig. 2 – Phylogenetic relationships of representative bacterial 16S rRNA gene sequences within the phylum Proteobacteria from clone libraries of this study determined by the neighbor-joining method. Bootstrap values of > 50% (obtained with 1000 resamplings) are shown at nodes. The scale bar indicates 0.05 nucleotide substitution per site. The reference sequences are obtained from the Ribosomal Database Project II or GenBank. *Methanobrevibacter ruminantium* is used as an outgroup. GenBank accession numbers are in parentheses.

river showed resistance to 80 µg/ml of oxytetracycline, while the resistance ratio was less than 5% in the reference upstream river. The significant difference of resistance ratios between the antibiotic containing water samples and the reference upstream river samples indicated that the concentrations of penicillin G and oxytetracycline residues in the polluted rivers and wastewater were high enough for leading most of the bacteria population in water samples resistant to antibiotics, in addition with the fact that the concentrations of 80 µg/ml of antibiotics tested in this study were much higher than the relevant breakpoints recommended by CLSI Standards guidelines (Clinical and Laboratory Standards Institute, 2003). The CFU number of ampicillin resistant bacteria was 3.4×10^4 cfu/ml in penicillin G containing downstream water, 4.8×10^4 cfu/ml in the aerobic effluent, and less than 8.9×10^2 cfu/ml in the reference upstream river water. Meanwhile the CFU number of oxytetracycline resistant bacteria in the oxytetracycline containing downstream river was 7.7×10^2 cfu/ml, and less than 1×10^2 cfu/ml in the reference upstream river.

3.2. 16S rRNA clone libraries and statistical analysis

Six clone libraries were individually constructed for the upstream and downstream water samples of both rivers, as well as the anaerobic and aerobic effluent of the WWTP for treating penicillin G production wastewater. Total of 355 sequences were obtained and grouped into 215 OTUs, representing 465 clones derived from the six clone libraries of this study (Table 1). Possible chimeras were discarded.

Several unique characteristics of the bacterial communities were observed for all antibiotics containing water samples, including both downstream rivers as well as the anaerobic and aerobic effluent, as illustrated in Fig. 1, of which dimension 1 explained 52.9% of the observed variation, and dimension 2 explained 21.3% of the variation. The most notable characteristics were the abundance of clones belonging to the deeply rooting classes *Deltaproteobacteria* and *Epsilonproteobacteria*, the total of which individually representing 15.9% and 40.3% of the clone numbers of the libraries for the penicillin G and oxytetracycline polluted downstream rivers, and accounting for 24.7% and 27.2% of the clones in the anaerobic and aerobic effluent libraries, respectively (Table 1). The clones grouped into *Deltaproteobacteria* in all antibiotic containing samples were mainly classified as *Desulfovibrio* spp., *Desulfovibrio mexicanus*, *Desulfovibrio desulfuricans*, *Desulfobacter* spp., *Desulfobacter postgatei*, *Desulfomicrobium norvegicum*, *Desulfomicrobium escambiense*, and *Desulfomicrobium apsheronum* (Fig. 2), all of which were sulfate- or sulfur-reducing bacteria. These species are generally strictly anaerobic and gain energy by coupling the complete or partial oxidation of organic compounds or molecular hydrogen to the reduction of sulfur or sulfate generating hydrogen sulfide. Then all clones in the class *Epsilonproteobacteria* were classified into the genera *Sulfurovum*, *Sulfurospirillum* and *Arcobacter*, all of which are sulfur and hydrogen sulfide- or thiosulfate-oxidizing bacteria, with nitrate or oxygen as electron acceptors.

Additionally, the phylum *Firmicutes* including the classes *Clostridia* and *Bacilli*, both of which were Gram-positive low GC

bacteria, had become the second abundant bacterial group comprising of 39.0% and 22.1% of the clones in the libraries for penicillin G and oxytetracycline polluted downstream rivers, respectively (Table 1). Meanwhile, the clones belonged to *Clostridia* and *Bacilli* were dominant in the anaerobic effluent library, and more or less as abundant as those belonging to *Proteobacteria* in the aerobic effluent library. The *Bacilli* clones in all these antibiotic containing water samples were further classified into the genera *Trichococcus* and *Streptococcus*, of which *Trichococcus* spp., especially *Trichococcus flocculiformis* were the dominant species among the *Bacilli* clones and appeared in almost all antibiotic containing samples (Fig. 3). *T. flocculiformis* is an aerotolerant, fermentative organism, and originally isolated from bulking sludge in Germany (Scheff et al., 1984). Furthermore, the majority of the clones (46.2%) in the class *Clostridia* have been classified into *Mitsuokella* spp. including *Mitsuokella multacida*. *M. multacida* was generally anaerobic rumen bacteria with phytase activity and was belonged to the *Sporomusa* subbranch of low GC Gram-positive bacteria. The remaining clones in the *Clostridia* were mainly affiliated with the anaerobic genera *Aminobacterium*, *Desulfosporosinus*, *Anaerovorax*, *Acetobacterium*, *Mogibacterium*, *Dialister*, *Clostridium*, *Thermoanaerobacterium* and *Ruminococcus*. Several genera were spore forming bacteria, such as *Desulfosporosinus*, *Clostridium* and *Thermoanaerobacterium*.

The similarities of OTUs among all the antibiotic containing environments were determined using software DOTUR. Totally, 13 OTUs were shared by the bacterial community libraries of the penicillin G polluted downstream river and the two penicillin wastewater samples, and 7 OTUs were shared by those of the oxytetracycline polluted downstream river and the two penicillin wastewater samples. Then 5 OTUs were shared by those of the two antibiotic containing downstream rivers, and 4 OTUs were shared by the two wastewater libraries. Two OTUs were furthermore shared across all antibiotic containing aquatic environments. Most of these OTUs fell into *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli*, also suggesting that these bacterial groups might be specifically associated with antibiotic containing aquatic environments at both species and classes levels. Several other bacterial groups seemed also to be related to the antibiotic containing aquatic environments, such as the class *Bacteroidetes*, as well as the phyla *Chloroflexi*, *Gemmatimonadetes*, and *Lentisphaerae* (Fig. 1 and Table 1). However, as few clones were obtained for these bacterial groups and distributed dispersedly in wastewater and downstream river samples, no more confirmative relationships could be drawn between these bacterial groups and antibiotic containing environments.

The bacterial community compositions in the two reference upstream river samples were also analyzed using clone libraries. Bacterial groups present were common residents in freshwater ecosystems, with the dominant groups as the phyla *Proteobacteria* including *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, as well as *Actinobacteria* and *Bacteroidetes* (Table 1). The remaining clones were classified into the phyla *Planctomycetes*, *Acidobacteria*, *Firmicutes* and *Verrucomicrobia*, present at much lower abundance. Only 1 OTU was shared by the bacterial communities of both upstream rivers. UniFrac metric analysis has demonstrated

Fig. 3 – Phylogenetic relationships of representative bacterial 16S rRNA gene sequences within the other phyla from clone libraries of this study determined by the neighbor-joining method. Bootstrap values of > 50% (obtained with 1000 resamplings) are shown at nodes. The scale bar indicates 0.05 nucleotide substitution per site.

Fig. 4 – Bacterial clone libraries clustering using UPGMA method to UniFrac metric based on all 16S rRNA gene sequences from the upstream and downstream clone libraries of the two rivers 1 and 2 individually receiving treated penicillin G and oxytetracycline production wastewater, as well as the anaerobic and the aerobic effluent clone libraries of penicillin G production wastewater.

that both bacterial community compositions in the upstream rivers were distinctly different from the remaining of the antibiotic containing aquatic environments (Fig. 4). PCoA analysis also revealed the similar results (data not shown).

Comparably higher richness and evenness indexes of the clone libraries derived from the aerobic effluent of the WWTP and the downstream river receiving penicillin G production wastewater were observed than those from the anaerobic effluent and the reference upstream river (Table 2), indicating that the bacterial communities in the aerobic effluent and the penicillin G polluted downstream river were more diverse. Meanwhile, the diversity of the clone library for the oxytetracycline containing downstream river was similar with that of the upstream river. It thus seems that the antibiotic stresses had not obviously reduced the diversity of bacterial community in aquatic environments. This result was accordant with that obtained before, in which phospholipid fatty acid numbers even increased in ciprofloxacin-treated microcosms (Córdova-Kreylos and Scow, 2007), while different from several recent studies on human gut microbiome which

showed that the diversity normally decreased significantly with the treatment of antibiotics (Antonopoulos et al., 2009; Rea et al., 2011). The comparably short exposure time of human gut microbiome should be the major reason for the significant reduce of bacterial diversity. It should be noted that as rarefaction curves of all clone libraries in this study did not reach saturation (data not shown), the clone number for each sample was still not sufficient and may affect the indices values.

4. Discussions

In this study, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* have been found abundant in all antibiotic containing environments. It has been reported that *Deltaproteobacteria* and *Firmicutes* were commonly abundant in the anaerobic wastewater treatment systems, with the clone percentages varied among different researches (Godon et al., 1997). Penicillin G production wastewater of this study was treated with a combination of anaerobic and aerobic processes. It is thus possible that the presence of *Deltaproteobacteria*, *Clostridia* and *Bacilli* in the aerobic effluent as well as the corresponding downstream river was related with the release from the anaerobic reactor. However, *Epsilonproteobacteria*, which is comparably uncommon in the anaerobic treatment systems reported previously (Godon et al., 1997), was the dominant *Proteobacteria* and accounted for 14.3% of the total clone number of the bacterial library for the anaerobic effluent. Meanwhile, many previous investigations have demonstrated that the phylum *Proteobacteria* was generally dominant in the aerobic reactors of WWTPs with *Betaproteobacteria* being the most frequently observed, and the phyla *Bacteroidetes* and *Actinobacteria* were frequently retrieved (Wagner and Loy, 2002). Then *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Acidobacterium* and *Bacteroidetes* were reported to account for the major proportion of the bacterial community in freshwater (Hugenholtz et al., 1998). Furthermore, the clones grouped in *Deltaproteobacteria*, *Clostridia* and *Bacilli* have totally accounted for 37.7% of the clone number of the library for the oxytetracycline containing downstream river, while no anaerobic treatment was adopted for the oxytetracycline wastewater of this study. Thus, the

Table 2 – Coverage and diversity indexes of bacterial 16S rRNA gene clone libraries for the upstream and downstream of the two rivers individually receiving treated penicillin G and oxytetracycline production wastewater, as well as the anaerobic and the aerobic effluent of penicillin G production wastewater.

Sample	No. of clones	No. of OTUs	S_{Chao1}	S_{ACE}	Shannon index	Evenness index	% Coverage
Upstream river1 ^a	75	23	38	33.87	2.82	0.899	86.7
Upstream river2 ^b	72	39	81.8	75.3	3.36	0.917	65.3
Downstream river1 ^a	82	45	85.6	118.82	3.52	0.925	64.6
Downstream river2 ^b	77	39	76.5	87.3	3.32	0.906	67.5
Anaerobic effluent	77	26	32.88	38.88	2.83	0.869	85.7
Aerobic effluent	81	43	116.5	118.84	3.5	0.931	63

a The river1 received treated penicillin G production wastewater.

b The river2 received treated oxytetracycline production wastewater.

unexpectedly high abundance of the bacterial groups including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* appeared in the aerobic effluent and both downstream rivers (the sampling sites generally 20 km away from the discharge points of wastewater) could not be only attributed to the release from the anaerobic treatment process. Furthermore, the abundance of these bacterial groups might be resulted from both the antibiotics and the co-existing pollutants in wastewater. However, the impacts of antibiotics should be much larger due to their strong bacteriostatic effects. By using molecular methods, several bacterial genera such as *Clostridium* spp., *Eubacterium* spp., *Streptococcus* spp. and *Lactobacillus* spp. belonging to the classes *Clostridia* and *Bacilli* in *Firmicutes* have been found sometimes still dominant in the human and poultry gut after feeding with antibiotics, together with *Bacteroides* spp. in *Bacteroidetes* (Knarreborg et al., 2002; Young and Schmidt, 2004; Jernberg et al., 2005). Antonopoulos et al. and Rea et al. have found that *Proteobacteria* is particularly enriched in human gut microbiome with the treatment of antibiotics (Antonopoulos et al., 2009; Rea et al., 2011). Lawrence et al. have observed a significant reduction in the abundance of *Betaproteobacteria* and *Gammaproteobacteria* in river biofilm communities exposed to a broad-spectrum antimicrobial chlorhexidine using fluorescent in situ hybridization (Lawrence et al., 2008), whereas no more information has been provided about the other bacteria groups in their research. Then in the study of Córdova-Kreylos and Scow (2007), sulfate-reducing bacteria belonged to *Deltaproteobacteria* including *Desulfovibrio*, *Desulfobacter* and *Desulfobulbus* were obviously favored by ciprofloxacin in the sedimental microbial communities. These previous reports have partially confirmed the specific association between the bacterial groups *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* and antibiotic containing environments.

It still need be noted that the penicillin G is grouped into β -lactam antibiotics and mainly active against Gram-positive bacteria by acting on bacterial cell wall, while oxytetracycline is a broad-spectrum antibiotic belonging to tetracyclines and inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Fluit et al., 2001). Several human infection cases caused by sulfate-reducing bacteria belonging to *Deltaproteobacteria* have been reported, and all of these sulfate-reducing strains had shown co-resistance to multiple antibiotics belonged to different classes (Pitcher et al., 1994; McDougall et al., 1997). Sulfate-reducing bacteria were also not affected by the administration of several different antibiotics in rats (Ohge et al., 2003). Several *Arcobacter* spp. belonging to *Epsilonproteobacteria* related to human infections had often been described to confer multi-drug resistance (Fera et al., 2003). In the classes *Clostridia* and *Bacilli*, tetracycline resistance gene *tet(W)* was first identified in one *M. multacida* isolate (Scott et al., 2000). Some isolates of *T. flocculiformis* in these classes had shown multiple antibiotic resistance abilities in our previous research (Li et al., 2009), and *Streptococcus bovis* has displayed multiple antibiotic resistance to several antibiotics (Teng et al., 2001), together with *Clostridium* spp. strains (Rood et al., 1978). The other bacterial genera in *Clostridia* and *Bacilli* including *Aminobacterium*, *Desulfosporosinus*, *Anaerovorax*, *Acetobacterium*, *Thermoanaerobacterium*, *Ruminococcus*, *Dialister* and *Mogibacterium* of this study are phylogenetically closely related to

intestinal bacterial groups including *Clostridium*, *Eubacterium*, *Streptococcus* and *Lactobacillus*, which were sometimes still dominant in the gut after feeding the antibiotics (Knarreborg et al., 2002; Young and Schmidt, 2004; Jernberg et al., 2005), suggesting that these bacterial genera of this study possibly shared similar antibiotic resistance mechanisms with those intestinal bacterial groups.

Several antibiotic resistance mechanisms have been described for bacteria, including antibiotic resistance genes which encode antibiotic modifying or inactivating enzymes and usually target one antibiotic class specifically, mutations of antibiotic target sites in bacterial cells, and efflux pump systems locating in bacterial cell membranes and mainly accounting for multi-drug resistance. The occurrence of the specific bacterial groups including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* in different antibiotics containing environments suggested that some kind of intrinsic resistance mechanisms might account for the wide-spread of these groups.

5. Conclusions

Several bacterial groups including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridia* and *Bacilli* have been suggested to be specifically associated with antibiotic containing aquatic environments, many of which have not been recovered in previous antibiotic resistance researches using culture-based analysis.

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