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Abundance and distribution of Macrolide-Lincosamide-Streptogramin resistance genes in an anaerobic-aerobic system treating spiramycin production wastewater

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ARTICLE INFO

Article history:

Received 15 January 2014

Received in revised form

20 May 2014

Accepted 24 May 2014

Available online 16 June 2014

Keywords:

Spiramycin wastewater

Anaerobic and aerobic biological treatment

Macrolide-Lincosamide-Streptogramin resistance genes

Mobile elements

ABSTRACT

The behaviors of the Macrolide-Lincosamide-Streptogramin (MLS) resistance genes were investigated in an anaerobic-aerobic pilot-scale system treating spiramycin (SPM) production wastewater. After screening fifteen typical MLS resistance genes with different mechanisms using conventional PCR, eight detected genes were determined by quantitative PCR, together with three mobile elements. Aerobic sludge in the pilot system exhibited a total relative abundance of MLS resistance genes (per 16S rRNA gene) 2.5 logs higher than those in control samples collected from sewage and inosine wastewater treatment systems ($P < 0.05$), implying the presence of SPM could induce the production of MLS resistance genes. However, the total relative gene abundance in anaerobic sludge (4.3×10^{-1}) was lower than that in aerobic sludge (3.7×10^0) despite of the higher SPM level in anaerobic reactor, showing the advantage of anaerobic treatment in reducing the production of MLS resistance genes. The rRNA methylase genes (*erm(B)*, *erm(F)*, *erm(X)*) were the most abundant in the aerobic sludge (5.3×10^{-1} – 1.7×10^0), followed by esterase gene *ere(A)* (1.3×10^{-1}) and phosphorylase gene *mph(B)* (5.7×10^{-2}). In anaerobic sludge, *erm(B)*, *erm(F)*, *ere(A)*, and *msr(D)* were the major ones (1.2×10^{-2} – 3.2×10^{-1}). These MLS resistance genes (except for *msr(D)*) were positively correlated with Class 1 integron ($r^2 = 0.74$ – 0.93 , $P < 0.05$), implying the significance of horizontal transfer in their proliferation.

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<http://dx.doi.org/10.1016/j.watres.2014.05.045>

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1. Introduction

Antibiotic production generates waste streams with high concentrations of chemical oxygen demand (COD_{Cr}), residual antibiotics and ammonium, which are, in general, treated by biological processes (Larsson et al., 2007; Ma et al., 2009). However, high antibiotic concentrations and bacterial densities in biological wastewater systems may favor the proliferation of antibiotic resistance genes (ARGs), which could pose health risks to humans via various pathways (Li et al., 2010; Munir et al., 2011; Zhang et al., 2013). Our previous study has revealed that large amounts of tetracycline resistance genes were generated during aerobic treatment of oxytetracycline (OTC) production waste stream (Liu et al., 2012). On the other hand, the anaerobic process has been widely used for the treatment of industrial wastewater containing high concentration of COD_{Cr} (Lettinga, 1995). At the same time, anaerobic digestion of excess sludge has been found to effectively reduce ARGs (Diehl and Lapara, 2010). However, the behaviors of ARGs during anaerobic wastewater treatment processes under high antibiotic levels have been seldom reported.

Macrolides, which could inhibit a wide range of bacteria, are a group of antibiotics frequently used in clinics and feed-lots (Iacoviello and Zinner, 2002). Macrolides share an overlapping binding site on bacterial ribosome with structurally distinct lincosamides and streptogramins, leading to clusters of genes encoding resistance to these antibiotics, called Macrolide-Lincosamide-Streptogramin (MLS) resistance genes (Sutcliffe and Leclercq, 2002). MLS resistance could be divided into three groups according to their resistance mechanisms: rRNA methylase genes, efflux genes, and enzymatic modification genes (Roberts et al., 1999). To date, 79 MLS resistance genes have been identified in a variety of gram-positive and gram-negative bacteria from human and animal origins (<http://faculty.washington.edu/marilynr/>). Recently, the occurrence of MLS resistance genes has gained increasing concerns. Chen et al. (2007) and Koike et al. (2010) surveyed rRNA methylase genes in swine feedlot environment and found that *erm*(B) and *erm*(F) had the highest relative abundance. Negreanu et al. (2012) reported that the relative abundances of *erm*(B) and *erm*(F) were at the levels of 10^{-4} – 10^{-3} in the raw influent and aerobic sludge of sewage treatment plants. However, the behaviors of MLS resistance genes during the treatment of macrolide production wastewater with high antibiotic levels have not yet been clarified.

In this work, we surveyed the occurrence, abundance, and distribution of MLS resistance genes in a pilot-scale biological system consisting of anaerobic, anoxic, and aerobic units in succession to treat spiramycin production waste stream (SPM wastewater) and its mixture with two other antibiotic production waste streams (paromomycin and ribostamycin) over a period of 9 months. Fifteen MLS resistance genes, which conduct different resistance mechanisms and have been reported in the environment previously, were screened by conventional PCR. Eight detected genes (*erm*(B), *erm*(F), *erm*(T), *erm*(X), *msr*(D), *mef*(A), *ere*(A), *mph*(B)) were determined using quantitative PCR (qPCR), together with three mobile elements (*int11*, *ISCR1*, and *Tn916/1545*). At the same time, clone libraries were constructed to analyze bacterial compositions and diversities of MLS resistance genes. This study will help understand the fate of ARGs during anaerobic and aerobic wastewater treatment of high antibiotic levels.

2. Materials and methods

2.1. Pilot-scale wastewater treatment systems and sampling

SPM is a 16-member macrolide widely used in veterinary clinics. Samples were taken from a pilot-scale SPM wastewater treatment system consisting of sequential up-flow anaerobic sludge bed (UASB), anoxic reactor, and oxic reactor (Fig. 1). The effective reactor volumes of the three reactors were 600 L, 200 L, and 400 L, respectively. The flow rate of raw wastewater was 200 L/d. The hydraulic retention time (HRT) was 48 h for UASB and oxic reactors, and 24 h for the anoxic reactor. The UASB effluent was recirculated to the inlet at a dilution ratio of 2:1 (flow rate ratio of UASB effluent to raw wastewater). The mixed liquor from the oxic reactor was recirculated to the anoxic reactor at a ratio of 2:1 (flow rate ratio of oxic effluent to UASB effluent). Sludge from the sedimentation tank was returned to the anoxic reactor at a ratio of 1:1. Sludge from a citrate production wastewater treatment plant and a sewage treatment plant was inoculated into the UASB reactor at a volume ratio of 1:1. Inoculum for the anoxic and oxic reactors (A/O reactors) was collected from a full-scale SPM wastewater treatment plant in Jiangsu Province. The operation of the system was divided into three stages: receiving (i) SPM wastewater, (ii) mixture of SPM wastewater and paromomycin wastewater, and (iii) mixture of SPM

Fig. 1 – Scheme of the SPM wastewater treatment systems and sampling sites. UASB: Up-flow Anaerobic Sludge Bed Reactor. W1, W2, W3, W4: Raw influent and effluent from UASB, Anoxic, and Aerobic reactors. S1, S2, S3: Sludge from the UASB, Anoxic, and Aerobic reactors, respectively. S1₁ – S3₁; S1₂ – S3₂; S1₃ – S3₃: sludge samples from the three stages treating spiramycin, spiramycin and paramomycin, spiramycin and ribostamycin, respectively.

wastewater and ribostamycin wastewater. Each stage lasted for three months.

Wastewater and sludge samples were collected to study the occurrence, abundance and distribution of MLS resistance genes in different treatment processes. Raw wastewater and effluent from each treatment unit (W1–W4) were collected every 6 h four times at the end of each stage, and nine sludge samples were collected from each reactor (S1–S3) once at the end of each stage (S₁–S₃, S₁–S₂, and S₁–S₃ for the three stages, respectively) (Fig. 1). The SPM fermentation residue (SPM-FR) was collected directly from the production facilities after SPM extraction and dehydration. For comparative study, two sludge samples, STP-AS (mixture of aerobic sludge from two sewage treatment plants located in Beijing and Tianjin, respectively) and Control-AS (sludge from an inosine production wastewater treatment system in Xinxiang City, Henan Province) were used as controls. All samples were transported to the lab at 4 °C and treated within 24 h.

2.2. Chemical analysis methods

COD_{Cr}, ammonia, total nitrogen, and total phosphate were determined according to the National Standard Methods of China (Table A1) (Wei, 2002). The concentrations of SPM in wastewater and sludge were determined using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS, Waters, USA), with detailed procedures described in Supplementary Information (Table A2).

2.3. DNA extraction

For each sludge sample, 1 mL of mixed liquor was centrifuged at 12,000 ×g for 10 min at 4 °C. Then 0.25 g sediment (wet weight) after centrifugation was used for DNA extraction by a FastDNA SPIN kit for soil (Qbiogene, Solon OH). The DNA solution volume was 80 µL, among which 2 µL was used for concentration determination by NanoDrop ND-1000 (USA) and 5 µL was checked by electrophoresis on a 1% (weight/volume) agarose gel.

2.4. PCR and quantitative PCR

Fifteen MLS resistance genes including eight rRNA methylase genes (*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), *erm*(Q), *erm*(T), *erm*(X)), three efflux genes (*msr*(A), *msr*(D), *mef*(A)), two esterase genes (*ere*(A), *ere*(B)), two phosphorylase genes (*mph*(A), *mph*(B)), and three mobile elements associated with MLS resistance genes (Class 1 integron (*intI1*), transposon Tn916/1545, the first group of insertion sequence common region (ISCR1)) were tested with primers listed in Table A3. The PCR assays were carried out for SPM-FR and all sludge samples from the SPM wastewater treatment systems, with sterile water used as the negative control. The PCR assays were conducted in 25 µL volume reactions using an ABI PCR System 9700 (ABI, USA). The PCR mixture consisted of 1 × Ex Taq 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 DNA. 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 (Table A3) and extension at 72 °C for

45 s, and finished with a final extension step at 72 °C for 7 min. PCR products were checked by electrophoresis on 1% (weight/volume) agarose gel in 1 × TBE buffer (Table A4). For each detected gene, the positive amplicons were further assured by cloning and sequencing.

Eight detected MLS resistance genes (*erm*(B), *erm*(F), *erm*(T), *erm*(X), *msr*(D), *mef*(A), *ere*(A), *mph*(B)), three detected mobile elements (*intI1*, ISCR1, Tn916/1545), and the 16S rRNA genes were quantified for all samples using SYBR-Green real-time qPCR, with the same primers as conventional PCR. Standard plasmids carrying target genes were obtained by TA clones and extracted using a TIANpure Mini Plasmid kit (Tiangen, China). Concentrations of the standard plasmids (ng µL⁻¹) were determined with the Nanodrop ND-1000 (Nanodrop, USA) and converted to the measurement of copies µL⁻¹ (Liu et al., 2012). 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 DNA templates. Real-time PCR was run using an ABI 7300 apparatus (ABI, USA) by the following program: 95 °C for 30 s, 40 cycles consisting of: (i) 95 °C for 10 s, (ii) annealing temperature for 15 s (Table A3), (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 ABI 7300 software. All standards, samples, and negative control (sterile water) were quantified in triplicates. 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 (Table A5): R² was higher than 0.99 for standard curves over 5 orders of magnitude. Amplification efficiencies based on slopes were between 90% and 110%. The specificity was assured by the melting curves and gel electrophoresis. The minimum quantification limits for all genes were in the range of 1.0 × 10² to 2.1 × 10³ copies per µL DNA.

The absolute abundances of MLS resistance genes and mobile elements (gene copies per sample volume) were shown in Table A6. To minimize variances caused by different bacterial abundances, DNA extraction and quantification efficiencies, the relative abundances of MLS resistance genes and mobile elements were obtained by normalizing their copy numbers to those of the 16S rRNA genes (Fig. 2, Table A7). There were no significant differences among the three treatment stages (*P* > 0.05), which made it possible to calculate the average relative abundances of MLS resistance genes using the data of the three stages (S₁_{mean}, S₂_{mean}, and S₃_{mean} for anaerobic, anoxic, and aerobic sludge, respectively) (Table A6 and A7).

2.5. Phylogenetic analysis of MLS resistance genes

Phylogenetic diversities of all detected MLS resistance genes were analyzed by cloning for the anaerobic or aerobic sludge from the SPM wastewater treatment system, using the same primers as conventional PCR. The diversities of *erm*(B), *ere*(A), and *msr*(D), which represent three different resistance mechanisms, were determined for both anaerobic and aerobic sludge. The diversity of *ere*(A), which showed the highest diversity in both aerobic and anaerobic sludge, was also investigated for sludge samples from the sewage and inosine wastewater treatment systems (STP-AS and Control-AS) for

Fig. 2 – Relative abundance of MLS resistance genes in anaerobic, anoxic, and aerobic sludge during three different stages treating SPM (S₁–S₃), SPM and paramomycin (S₁–S₂), SPM and ribostamycin (S₁–S₃). MLS^T: the total abundance of all determined MLS resistance genes.

comparison. The cloning process was the same as previously described (Liu et al., 2012). Sequences with identities lower than 98% (calculated by the software Lasergene version 7 (DNASTar, USA)) were clustered as different gene groups (Koike et al., 2007). One representative sequence was chosen

from each group to construct neighbor-joining phylogenetic trees using the software Mega version 4.1 (<http://megasoftware.net/>), together with sequences retrieved from NCBI GenBank (Fig. 3 and A2). The phylogenetic distributions of *ere(A)* in different systems are shown in Table 1. The MLS resistance gene sequences were deposited in NCBI GenBank under accession No. KC669391 to KC669407.

2.6. Statistical analyses

All statistical analyses were performed using SPSS version 15.0. Paired samples t-tests and P-values were used to assess significant differences between systems and samples. Pearson's bi-variate correlation analysis was used to assess correlations between MLS resistance genes and mobile elements in sludge samples from SPM wastewater treatment systems.

3. Results

3.1. Chemical characteristics of wastewater and sludge

As shown in Tables A1 and A2, the system exhibited 91.3% COD_{Cr} removal and 92.7–97.1% SPM removal at three different stages. The concentration of COD_{Cr} was 7193.4 ± 424.3 mg/L in the influent, which decreased to 1431.2 ± 56.4 mg/L, 1000.0 ± 35.4 mg/L and 615.2 ± 62.7 mg/L following anaerobic, anoxic and aerobic treatment, respectively. The concentrations of SPM were at high levels (12.4–41.8 mg/L) in the influent (W1), and decreased to 1.8–3.8 mg/L and 0.9–3.0 mg/L after anaerobic (W2) and aerobic (W4) treatment, respectively. The SPM concentrations in sludge (S1–S3) were 110.3–289.4 mg/kg dry weight when influent (W1) SPM level was 41.0 mg/L, and 24.5–149.6 mg/kg dry weight when influent (W1) SPM level was 12.4 mg/L. The SPM concentration in the fermentation residue (SPM-FR) was 931.3 mg/kg dry weight.

3.2. Occurrence of MLS resistance genes

As shown in Table A4, eight of the fifteen MLS resistance genes were detected in the sludge samples of the SPM wastewater

Fig. 3 – Neighbor-joining phylogenetic tree of *ere(A)* in sludge samples from SPM wastewater, sewage, and inosine wastewater treatment systems.

Table 1 – Phylogenetic distribution of *ere(A)* in sludge samples from SPM wastewater (S1 and S3), sewage (STP-AS), and inosine production wastewater treatment systems (Control-AS).

Samples	Clones of each sequence type									
	Typ1	Typ2	Typ3	Typ4	Typ5	Typ6	Typ7	Typ8	Typ9	Typ10
S1	2	20	1	1	–	–	–	–	–	–
S3	16	4	–	–	1	–	–	1	1	–
STP-AS	20	2	–	–	–	1	1	–	–	–
Control-AS	18	–	–	–	–	–	–	–	–	1

“–”: not detected.

treatment system, including four rRNA methylases (*erm(B)*, *erm(F)*, *erm(T)*, *erm(X)*), two efflux proteinase genes (*mef(A)*, *msr(D)*), one esterase gene *ere(A)*, and one phosphorylase gene (*mph(B)*). The rRNA methylase genes *erm(B)* and *erm(F)*, and the esterase gene *ere(A)* were the most prevalent resistance genes detected in all sludge samples. rRNA methylase gene *erm(X)* and phosphorylase gene *mph(B)* were detected in all aerobic sludge, but less frequent in anaerobic sludge (2/3 and 1/3, respectively). The rRNA methylase gene *erm(T)* and the efflux genes *mef(A)* and *msr(D)* were more frequently detected in the three anaerobic sludge (S1: 2/3 – 3/3) than those in the aerobic sludge (S3: 0/3). *int1* was detected in all sludge samples, while Tn916/1545 was more frequently detected in anaerobic sludge samples (S1, 3/3) than that in aerobic sludge (S3, 2/3). No MLS resistance genes or mobile elements were detected by conventional PCR in the SPM fermentation residue (SPM-FR).

3.3. Relative abundance of MLS resistance genes and mobile elements

As shown in Table A7, only *erm(B)*, *erm(F)*, *erm(T)*, *erm(X)*, and *msr(D)* were detected in SPM-FR with relative abundances between 7.0×10^{-6} to 1.5×10^{-3} , which totally (MLS^T) added up to 1.6×10^{-3} . The relative abundances of MLS resistance genes in anaerobic sludge ($S1_{\text{mean}}, 1.0 \times 10^{-4}$ – 3.2×10^{-1}) and aerobic sludge ($S3_{\text{mean}}, 2.4 \times 10^{-4}$ – 1.7×10^0) were much higher than those in SPM-FR. By comparison, the relative abundances of the MLS resistance genes in the aerobic sludge samples from sewage and inosine wastewater treatment systems were much lower (STP-AS: undetectable– 9.5×10^{-3} , Control-AS: undetectable– 8.2×10^{-3}). Since the inoculum for anaerobic treatment was collected from citrate wastewater and sewage treatment plants, it's assumed that the relative abundance of MLS resistance genes in the anaerobic seed sludge was comparable to that in the control systems.

Results showed that *int1* was abundant in aerobic sludge ($S3_{\text{mean}}, 1.3 \times 10^0$), but less abundant in anaerobic sludge ($S1_{\text{mean}}, 1.2 \times 10^{-2}$). The relative abundances of Tn916/1545 and ISCR1 were 8.8×10^{-2} and 4.6×10^{-5} in anaerobic sludge and 1.5×10^{-2} and 1.4×10^{-3} in aerobic sludge. Both *int1* and Tn916/1545 were at low levels (2.0×10^{-6} and 1.8×10^{-4} , respectively), and ISCR1 was below the detection limit in SPM-FR.

3.4. Cloning of bacterial 16S rRNA genes

As shown in Table A8, gram-positive bacteria mainly composed of Firmicutes, were dominant in anaerobic sludge

(S1), accounting for 77.1% of the total bacteria. However, gram-negative bacteria, which were mainly composed of Alpha-proteobacteria (18.5%) and Beta-proteobacteria (33.3%), became dominant (96.2%) in aerobic sludge (S3). All OTUs from the SPM-FR clone library were 99.6–100.0% identical to *Streptomyces ambofaciens*, the reported production bacteria of SPM (Sutcliffe and Leclercq, 2002).

3.5. Diversity of MLS resistance genes

The phylogenetic trees of all eight detected MLS resistance genes in sludge samples of the SPM wastewater treatment system are shown in Fig. 3 and Fig. A2. *ere(A)* exhibited the highest sequence diversity, with 10 different types (Typ1–Typ10) detected (Table 1). Typ1 with 99% identity to *ere(A)* in *Proteus mirabilis* (JX867129) and *Escherichia coli* plasmid (AY183453) was the most abundant in all aerobic sludge samples (16/23, 20/24 and 18/20 in S3, STP-AS and Control-AS, respectively). Typ2, which was 99% identical to *ere(A)* in *Providencia stuartii* plasmid (AF099140) and *Enterobacter aerogenes* (AF326209), was the most abundant in the anaerobic sludge samples (S1, 20/24). Typ3 to Typ10, which were >95.5% identical to known *ere(A)* sequences, were sparsely distributed in different samples (only one clone for each type).

Conserved sequences of *erm(B)* with over 99% identity to those in *Enterococcus faecalis* (M11180) and *Clostridium difficile* (AJ294530) were found in anaerobic and aerobic sludge samples (S1 and S3), consistent with previous research showing that all *erm(B)* sequences obtained from swine lagoons and groundwater were over 99% identical to each other (Koike et al., 2010). For *msr(D)*, most sequences (Typ1, 39/42) were identical to each other (99.3%–100.0%) in anaerobic and aerobic sludge (S1 and S3), while three different sequences (Typ2) with lower identities (95.6%–96.2%) occurred only in anaerobic sludge. All *msr(D)* sequences were 96–97% identical to those reported in the genome and transposons of *Streptococcus*, *Bacteroides*, *Gardnerella*, and *Clostridium*, etc. The other MLS resistance genes were very conservative in the aerobic sludge of the SPM wastewater treatment system, with high identities to each other (>99%).

4. Discussion

Till now, there have been a few reports about MLS genes in the environment. Chen et al. (2007) found that the relative abundances of *erm(B)*, *erm(F)*, *erm(T)*, and *erm(X)* were in the range of 4.4×10^{-4} – 5.4×10^{-2} and 7.4×10^{-5} – 4.3×10^{-1} in swine

manures and lagoons, respectively. Koike et al. (2010) reported a similar relative abundance of *erm*(B) (4×10^{-4} – 10^{-2}) in swine lagoons. Marti et al. (2013) reported that the relative abundance of *erm*(B) was at the magnitude of 10^{-4} – 10^{-3} in the biofilm and sediments of a sewage treatment system. Stoll et al. (2012) even found high prevalence of *erm*(B) in surface water from Germany and Australia. The relative abundances of MLS resistance genes in the SPM wastewater treatment system were higher than those in the above systems. According to the results in this study, the total relative abundance of MLS resistance genes in the SPM wastewater treatment system was 1.4–2.3 logs higher than those in control systems and the SPM fermentation residue. Similarly, our previous work found more abundant *tet* genes in an oxytetracycline wastewater treatment system than those reported in sewage treatment systems (Liu et al., 2012). Compared to OTC, SPM with higher molecular weight ($MW_{\text{SPM}} = 843.0$, $MW_{\text{OTC}} = 460.4$) is more difficult to attack enteric and other gram-negative bacteria because their outer cell membrane acts as a strong permeability barrier (Iacoviello and Zinner, 2002). However, MLS resistance genes were obviously selected in aerobic sludge that was dominated with gram-negative bacteria.

As a matter of fact, six of the eight detected MLS resistance genes (except for *erm*(T) and *erm*(X)) have been frequently reported in a wide range of gram-negative bacteria from human and animal origins (<http://faculty.washington.edu/marilynr/>). It was even reported that MLS genes occurred more frequently in gram-negative bacteria (78%) than in gram-positive bacteria (36%) isolated from healthy population exposed to low antibiotic levels (Luna et al., 2002; Ojo et al., 2004). Horizontal gene transfer among gram-positive and gram-negative bacteria might be partly responsible for the high abundance of MLS resistance genes in aerobic sludge dominated with gram-negative bacteria (Courvalin, 1994; Gupta et al., 2003; Ojo et al., 2006). Considering that SPM could induce significant accumulation of ARGs in aerobic sludge systems, excess sludge generated during antibiotic production wastewater treatment should be disposed carefully to control the spread of ARGs in the environment.

In aerobic sludge, the rRNA methylase genes *erm*(B), *erm*(F), and *erm*(X) were the most abundant, followed by enzymatic modification genes *ere*(A) and *mph*(B), with efflux genes *mef*(A) and *msr*(D) being the least abundant. In anaerobic sludge, *erm*(B), *erm*(F), *ere*(A), and *msr*(D) were the major ones. Chen et al. (2007) and Koike et al. (2010) both found that *erm*(B) and *erm*(F) were the most abundant rRNA methylase genes in swine manure and lagoons, mainly attributed to their wide host range (34 and 24 genera, respectively) and frequent association with mobile elements (Roberts, 2004). It is noteworthy that a high relative abundance of *erm*(X) was observed in aerobic sludge, despite its limited host bacteria (6 genera affiliated to Actinobacteria) (<http://faculty.washington.edu/marilynr/>) and low abundance in swine manure and lagoons (Chen et al., 2007), implying that SPM wastewater treatment system contained more diverse host bacteria for *erm*(X) than other environments.

The esterase gene *ere*(A) and phosphorylase gene *mph*(B), which are mainly harbored by gram-negative bacteria (<http://faculty.washington.edu/marilynr/>), showed high abundances

in aerobic sludge. Esterase genes in *Enterobacteria* and *Pseudomonas* led to SPM resistance and could biodegrade erythromycin by hydrolysis of the lactone ring (Arthur et al., 1987; Kim et al., 2004). *mph*(B) was reported to inactivate both 14-membered and 16-membered macrolides by phosphorylation of the 2-hydroxyl group of amino sugar (Achard et al., 2008; Chesneau et al., 2007). Thus, the high abundance of these genes indicated possible biodegradation of SPM.

It is believed to be cost-efficient for bacteria to evolve membrane transporters to achieve multi-drug resistance (Martinez et al., 2009). Our previous study showed the dominance of efflux *tet* genes (*tet*(A), *tet*(C), and *tet*(G)) in aerobic sludge from OTC wastewater treatment systems (Liu et al., 2012). However, efflux MLS resistance genes *mef*(A) and *msr*(D), which often occur together in a wide range of host bacteria (28 and 22 genera, respectively, among which 21 and 17 genera belong to gram-negative bacteria), showed unexpectedly low abundance in the aerobic sludge from the SPM wastewater treatment system. Different distributions of MLS resistance genes and *tet* genes may be partly attributed to the different chemical structures and molecular weights of SPM and OTC.

The most interesting result was that the relative abundances of most MLS resistance genes were lower in the anaerobic sludge than those in the aerobic sludge, although anaerobic sludge was exposed to higher SPM levels. Similar conditions were found in sludge digestion, where anaerobic digestion outperformed aerobic sludge in reducing tetracycline resistance genes and Class 1 integron (Diehl and Lapara, 2010). ARGs proliferate by vertical evolution and horizontal transfer. The slow bacterial growth rate and low biomass yield in anaerobic sludge (Lettinga, 1995) could restrict the mutation during cell reproduction (i.e. vertical evolution). As for gene acquisition by horizontal transfer, the relative abundances of mobile elements (*int*I1 and ISCR1) in anaerobic sludge were approximately two orders lower than those in aerobic sludge (Table A7), which means gene transfer in anaerobic sludge may not be as significant as that in aerobic sludge. Similarly, Rysz et al. (2013) reported that higher oxygen availability enhanced resistance plasmid (another sort of mobile element) maintenance in pure cultured bacteria because aerobic condition eased the metabolic burden of plasmid reproduction. Less accumulation of ARGs in anaerobic sludge than in aerobic sludge suggests the merit of employing anaerobic digestion in treating antibiotic production wastewater, though the mechanism requires more study.

Significant positive correlations were found between *int*I1 and five most abundant MLS resistance genes (*erm*(B), *erm*(F), *erm*(X), *ere*(A), and *mph*(B)) for all sludge samples in the SPM wastewater treatment system ($r^2 = 0.74, 0.92, 0.97, 0.88, 0.93$; $P < 0.05$) (Fig. 4), indicating the important roles of *int*I1 in their proliferation. Similar conditions were reported between *int*I1 and tetracycline and sulfonamide resistance genes (Liu et al., 2012; Wang et al., 2014). *int*I1, which integrates and transfers ARGs via gene cassettes, is the most prevalent mobile element in all kinds of environments (Gillings et al., 2008). *ere*(A) was reported as the gene cassette of *int*I1 in *Vibrio cholera* (Thungapathra et al., 2002). Though other MLS resistance genes were seldom found as gene cassettes of *int*I1, *int*I1 could promote their proliferation in other ways. Inserting sequence common regions (ISCR) is a newly highlighted complex

Fig. 4 – Correlations between MLS resistance genes and *intI1* in sludge samples from SPM wastewater treatment systems.

integron element which can integrate non-cassette resistance genes, in which an *erm* gene was reported (Liu et al., 2012; Toleman et al., 2006). ISCR1, the first group of ISCR, was quantified and showed positive correlations with *erm*(F), *erm*(X), and *mph*(B) ($r^2 = 0.65, 0.68, 0.71, P < 0.05$) (Fig. A3). Still, direct demonstration of the potential roles of *intI1* and ISCR in the proliferation of these ARGs is required.

According to the phylogenetic analysis of the most diverse gene *ere*(A), activated sludge samples from SPM wastewater, sewage, and inosine wastewater treatment systems shared the same dominant gene type of *ere*(A), implying that different SPM selection pressures did not impact the phylogenetic distribution of MLS resistance genes though it impacted their abundances. However, anaerobic sludge had a different dominant *ere*(A) gene type, which may be attributed to different host bacteria and different accumulation pathways therein.

5. Conclusion

In this study, MLS resistance genes were investigated in a biological SPM wastewater treatment system with anaerobic and aerobic units. The SPM wastewater treatment system produced significantly more MLS resistance genes than other systems both in this study and in previous reports, suggesting SPM wastewater treatment system is another important source of ARG pollution. The total relative abundance of MLS resistance genes was lower in anaerobic sludge than that in aerobic sludge, suggesting the merit of enhancing anaerobic digestion during antibiotic wastewater treatment. The most abundant MLS resistance genes in the aerobic sludge were rRNA methylase genes *erm*(B), *erm*(F), and *erm*(X), followed by esterase gene *ere*(A) and phosphorylase gene *mph*(B), while the

major genes in anaerobic sludge were *erm*(B), *erm*(F), *ere*(A), and *msr*(D). All the above genes except for *msr*(D) were positively correlated with *intI1*, implying the potential role of horizontal transfer in their proliferation. Different phylogenetic distributions of *ere*(A) were found in anaerobic and aerobic sludge, implying that MLS resistance genes may proliferate in different mechanisms under anaerobic and aerobic environment.

Acknowledgments

This study was supported by the National Natural Scientific Foundation of China (NSFC–21277162) and the Ministry of Science and Technology, the People's Republic of China (2012AA063401 and 2013DFG50150). We thank Ms. Li Mingyue for her kind help in sampling.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2014.05.045>.

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