Changes of resistome, mobilome and potential hosts of antibiotic resistance genes during the transformation of anaerobic digestion from mesophilic to thermophilic

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ABSTRACT

This study aimed to reveal how antibiotic resistance genes (ARGs) and their horizontal and vertical transfer-related items (mobilome and bacterial hosts) respond to the transformation of anaerobic digestion (AD) from mesophilic to thermophilic using one-step temperature increase. The resistomes and mobilomes of mesophilic and thermophilic sludge were investigated using metagenome sequencing, and the changes in 24 representative ARGs belonging to three categories, class 1 integron and bacterial genera during the transition period were further followed using quantitative PCR and 454-pyrosequencing. After the temperature increase, resistome abundance in the digested sludge decreased from 125.97 ppm (day 0, mesophilic) to 50.65 ppm (day 57, thermophilic) with the reduction of most ARG types except for the aminoglycoside resistance genes. Thermophilic sludge also had a smaller mobilome, including plasmids, insertion sequences and integrons, than that of mesophilic sludge, suggesting the lower horizontal transfer potential of ARGs under thermophilic conditions. On the other hand, the total abundance of 18 bacterial genera, which were suggested as the possible hosts for 13 ARGs through network analysis, decreased from 23.27% in mesophilic sludge to 11.92% in thermophilic sludge, indicating fewer hosts for the vertical expansion of ARGs after the increase in temperature. These results indicate that the better reduction of resistome abundance by thermophilic AD might be associated with the decrease of both the horizontal and vertical transferability of ARGs.

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1. Introduction

Many studies have demonstrated that sewage sludge from municipal wastewater treatment plants (WWTPs) is an important reservoir for antibiotic resistance genes (ARGs) (Auerbach et al., 2007; Munck et al., 2015). For example, six tetracycline resistance genes (*tet(A)*, *tet(C)*, *tet(G)*, *tet(M)*, *tet(S)*, and *tet(X)*) have been detected in the sewage sludge of 15 WWTPs from different regions (Zhang and Zhang, 2011), and 140 antibiotic resistance

determinants including the recently described clinically relevant resistance genes (*aadA6*, *imp-9*, *qnrB1*, etc.) were identified in the plasmid metagenome of activated sludge bacteria (Szczepanowski et al., 2009). Thus, excess sewage sludge should be handled properly to prevent the release of ARGs into the environment.

Recently, the effectiveness of anaerobic digestion (AD), which is a widely applied technology for the disposal of excess sludge (Appels et al., 2008), in mitigating the quantities of ARGs in sewage sludge was also noticed. Using quantitative polymerase chain reaction (qPCR), Ghosh et al. (2009a) found that full-scale thermophilic digestion showed better reduction in three tetracycline resistance genes (*tet(A)*, *tet(O)*, and *tet(X)*), and class 1 integron (*int11*), an important mobile element for horizontal ARG transfer, than the more widely applied mesophilic digestion. Similar results were confirmed in lab-scale studies by Diehl and LaPara (2010).

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However, subsequent research targeting other resistant determinants found that the benefits of thermophilic digestion might not be universal (Ma et al., 2011). Recently, metagenomic analysis was applied and revealed that thermophilic sludge had a slightly lower resistome than that of mesophilic sludge (Zhang et al., 2015). Recent studies also showed that the combination of metagenomics and qPCR might provide a more comprehensive and quantitative insight into the dynamics of microbial population and ARGs during wastewater and sludge treatment (Aydin et al., 2015; Zhang et al., 2015; Ju et al., 2016).

Despite the above efforts, the mechanisms behind the reduction of ARGs during mesophilic and thermophilic processes are not well explored or understood. ARGs proliferate mainly through two processes: vertical gene transfer attributed to the reproduction of bacterial hosts, and horizontal gene transfer attributed to the transfer of ARGs between different bacterial cells via mobile elements (Sorensen et al., 2005). Because of the decrease of *int1* during AD, the reduction of ARGs has been speculated to be associated with the blockage of their horizontal transfer pathways (Diehl and LaPara, 2010; Ma et al., 2011). Although suggested as an indicator of horizontal gene transfer potential (Ma et al., 2011), *int1* is not mobile in its own right and does not contain many important ARGs such as *tet* genes (Toleman et al., 2006). Thus, other mobile elements including plasmids and transposons should be investigated to validate the speculation. On the other hand, recent studies have shown that bacterial communities structured soil and sludge resistomes (Forsberg et al., 2014; Su et al., 2015). Considering the completely different bacterial populations in mesophilic and thermophilic digestion sludge (Ma et al., 2011; Tian et al., 2015), disturbance of the vertical transfer pathway might also be an important factor of ARG reduction. Thus, simultaneously exploring the responses of ARGs and their horizontal and vertical transfer-related items (mobilome and bacterial hosts) to shifts in digestion temperature might be helpful in revealing the mechanisms of ARGs reduction.

In our previous study, an anaerobic thermophilic digester was successfully started up to treat excess sewage sludge from a mesophilic one using the strategy of one-step temperature increase (Tian et al., 2015). Here, metagenomics and quantitative PCR were jointly used to reveal the comprehensive profiles of ARGs in digested sludge before and after temperature increase and the fates of 24 antibiotic resistance determinants and *int1* over the transition period. Changes in mobilome, distribution of ARGs in plasmids and co-occurrence patterns between ARGs and bacterial taxa were also analyzed. This work provides scientific support for controlling the spread of ARGs in the environment.

2. Material and methods

2.1. Reactor setup and transition from mesophilic to thermophilic AD

This experiment was carried out in one completely stirred tank reactor (CSTR) with 8 L total volume and 6 L working volume. The CSTR was operated at a constant feed rate of 300 mL/day and solid retention time (SRT) of 20 days. Substrate, a mixture of primary and secondary sludge collected from Gaobeidian WWTP, was fed into the reactor once a day. The reactor was operated under mesophilic conditions (35 °C) for more than 100 days before the temperature was increased to 55 °C in one step and then maintained under thermophilic conditions for two months. The detailed operation conditions of the reactor and the anaerobic digestion performance are described in previous research (Tian et al., 2015).

2.2. Sample collection and DNA extraction

Digested sludge samples of Day 0, 4, 11, 18, 26, 34, 44, and 57 after temperature increase and excess sewage sludge samples of Day 0, 11, 34 and 57 were taken from the reactor for DNA extraction. Each sludge sample (1 mL) was centrifuged at 10,000 rpm for 10 min at 4 °C, and the resulting sediment was used for DNA extraction using FastDNA® SPIN kit for soil (Qbiogene, Solon OH, USA) according to the manufacturer's instructions. For each sample, DNA was extracted in duplicate to avoid bias, and extracts from the same sample were pooled. Concentrations and quality of the extracted DNA were checked by spectrophotometric analysis on a NanoDrop ND-1000 (Nanodrop, USA) and electrophoresis on a 1% (weight/volume) agarose gel. Due to the stable composition of the sewage sludge over the experimental period (data not shown), four DNA samples of sewage sludge were mixed equally for subsequent molecular analysis.

2.3. 454 pyrosequencing of bacterial communities

For 454-pyrosequencing, bacterial 16S rRNA genes in nine sludge samples were amplified with primers 27F (5'-AGAGTTT-GATCCTGGCTCAG-3') and 534R (5'-TTACCGCGGCTGCTGGCAC-3') (Koike et al., 2007). Barcodes that allow sample multiplexing during pyrosequencing were incorporated between the 454 adapter and the forward primers. The length of the amplicon, including the barcode and 454 primers, was ~596 nt. The PCR amplification system and program are described in detail in previous work (Tian et al., 2015). The PCR products were purified and then sequenced by pyrosequencing using a 454 Life Sciences Genome SequencerFLX (Roche Diagnostics, Indianapolis, IN, USA). Raw pyrosequencing data were deposited in the NCBI Sequence Read Archive with accession no. SRP049225 (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP049225>). After sequencing, the sequence reads were analyzed using Mothur software (Tian et al., 2015).

2.4. Quantitative PCR assay

Nine tetracycline resistance genes including four efflux genes (*tet(A)*, *tet(C)*, *tet(G)*, *tet(L)*), four ribosomal protection protein genes (*tet(Q)*, *tet(O)*, *tet(M)*, *tet(W)*) and one inactivating enzyme gene (*tet(X)*), seven macrolide resistance genes including four rRNA methylase genes (*erm(B)*, *erm(F)*, *erm(T)*, *erm(X)*), one efflux gene (*mef(A)*), one esterase gene (*ere(A)*) and one phosphorylase gene (*Mph(B)*), eight aminoglycoside resistance genes including two acetyltransferases genes (*aac(3)-II*, *aacA4*), three nucleotidyl-transferase genes (*aadA*, *aadB* and *aadE*), and three phosphotransferases genes (*aphA1*, *strA* and *strB*), one mobile element (*int1*) and the 16S rRNA gene for domain bacteria were quantified using SYBR-Green real-time qPCR. The specificities of the used primer sets (Table S1) have been validated in recently published papers (Ma et al., 2011; Liu et al., 2012, 2014; Aydin et al., 2015) and verified in this study. 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) were determined with the NanoDrop ND-1000 (Nanodrop, USA), and their copy concentrations (copies/μL) were then calculated (Pei et al., 2006). The 25 μL reactions typically contained 1 × Sybr Green I, 1 × Dye (Takara), 200 nM each primer, 0.5 mg/mL 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 S1), (iii) 72 °C for 15 s, and (iv) 78 °C for 26 s to collect the fluorescent signals. The melting process was automatically

generated using ABI 7300 software. Serial dilutions of the standard plasmids were used as calibration standards. Standard curves were constructed in each PCR run and the copied numbers of the genes in each sample were interpolated using these standard curves. All standards, samples, and negative control (sterile water) were quantified in triplicate. 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 S2): R^2 was higher than 0.99 for standard curves over five 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 detection limits for all genes were in the range of 1.1×10^2 to 1.4×10^3 copies per μL DNA. To minimize the variances caused by differences in bacterial abundance, DNA extraction and quantification efficiencies, relative abundances of ARGs and *int11* were used by normalizing their copy numbers to those of the bacterial 16S rRNA genes for comparison (Figs. S1–S3, Table S3). Absolute abundance on a per sludge volume basis is shown in Table S4.

2.5. Network analysis

To visualize the correlations between ARGs and bacterial taxa, 24 ARGs quantified by qPCR and 474 bacterial genera identified by pyrosequencing were combined, and a co-occurrence network was constructed by the random matrix theory-based network inference method across eight digested sludge samples (Deng et al., 2012). First, a correlation matrix was constructed by calculating all possible pairwise Pearson correlations between the 498 items that occurred in at least five samples. The correlation matrix was then converted into the similarity matrix by taking absolute values (Horvath and Dong, 2008). Thereafter, a series of thresholds from 0.30 to 1.00 with 0.01 intervals were applied to the matrix, and only the similarity values above a certain threshold were kept for calculating matrix eigenvalues. The finest threshold was chosen when the nearest neighbour spacing distribution of eigenvalues followed Poisson distribution, which represents specific and non-random properties of complex systems (Deng et al., 2012), and meanwhile the threshold should be as low as possible (Deng et al., 2016). Network analyses were performed using an online analysis pipeline at <http://ieg2.ou.edu/MENA>. Network visualization was conducted on the interactive platform of Cytoscape (version 3.2.0).

2.6. Illumina high-throughput sequencing

Approximately 5 μg of DNA of digested sludge samples on day 0 (Meso-d0) and day 57 (Thermo-d57) was used for library construction and sequencing. High-throughput sequencing was conducted at Novogene (Beijing, China) using Illumina Hiseq 2500 with the sequencing strategy of index PE125+8+125 cycle (Paired-End sequencing, 125-bp reads and 8-bp index sequence). Approximately 5 Gb of data was generated for each sample. The raw Illumina read data of the two samples has been deposited in the European Nucleotide Archive, under the accession PRJEB13054 (<http://www.ebi.ac.uk/ena/data/view/PRJEB13054>). An online tool kit (Galaxy, <https://usegalaxy.org/>) was utilized to filter low quality reads to ensure (1) less than three ambiguous nucleotides in one read; (2) more than 90% bases with quality score greater than 30; and (3) no sequencing artifacts (Miao et al., 2015).

A local database of resistances genes was created by downloading all sequences from the Antibiotic Resistance Genes Database (ARDB) (<http://ardb.cbcb.umd.edu/>), and the redundant sequences from the downloaded database were removed using a customized script as well as manual checking. The nonredundant ARDB contains about 3000 sequences. All quality-filtered reads of

the two samples were searched for ARGs against the nonredundant ARDB using BLASTx with E-value $\leq 10^{-5}$ (Yang et al., 2013). A read was annotated as an ARG-like fragment according to its best BLASTx hit if the hit had a sequence similarity of above 90% over an alignment of at least 25 amino acids (Kristiansson et al., 2011). Local databases of insertion sequences (ISs), integrons and plasmids were separately created by downloading ISs sequences from the ISfinder (<https://www-is.biotoul.fr/>), integrase genes from the INTEGRALL (<http://integrall.bio.ua.pt/>) and plasmid sequences from the NCBI plasmid genome database (<ftp://ftp.ncbi.nih.gov/genomes/Plasmids/>). Sequences belonging to plasmid, integron- or IS-like sequences were identified by aligning the sequences against the three mobile genetic elements databases using BLASTx with an E-value cut-off of 10^{-5} and then filtered using the same conditions as ARG identification. The levels of ARGs or mobile element-like sequences in the present study were described using the unit of “ppm” (one read in one million reads) as recommended by Yang et al. (2013), which was defined as the portion of ARG or mobile element-like sequences in “total metagenome sequences”.

To explore the distribution of ARGs on plasmids, all clean reads were assembled using Velvet with 51-subunit Kmers. The obtained contigs of each sludge sample were aligned using BLASTx and compared with ARDB using an E-value $\leq 10^{-5}$, and filtered using cut-offs at identity $\geq 90\%$ and alignment length ≥ 25 aa. Then, the contigs that carried ARG-like fragments were further aligned against the local plasmid genome database using BLASTn with E-value $\leq 10^{-5}$ and then filtered using the conditions described above.

2.7. Statistical analysis

Pair-sample *t*-tests were conducted using Origin 8.0 to determine whether qPCR-based ARGs and *int11* abundances were significantly different between samples. A *p*-value of <0.05 was considered significant. Pearson's bi-variate correlation analysis was performed using SPSS 15.0 to assess correlations between ARGs and *int11*.

3. Results

3.1. Resistomes and mobilomes of digested sludge before and after temperature increase

As described in our previous study, the thermophilic microbial community was established in 20 days after the increase in temperature from 35 °C to 55 °C (Tian et al., 2015). So, two digested sludge samples, one representing the mesophilic community and another representing the thermophilic community, were taken on day 0 and day 57, respectively. The ARG profiles of the two samples were revealed using the metagenomic approach, as shown in Fig. 1. A total of 13 types of ARGs were detected in mesophilic sludge (day 0), with 9 types of ARGs having an abundance over 1 ppm. Tetracycline (47.96 ppm), sulfonamide (23.09 ppm) and macrolide-lincosamide-streptogramin (MLS) (13.88 ppm) resistance genes were the three dominant types in mesophilic sludge, followed by those for aminoglycoside (12.73 ppm), chloramphenicol (9.09 ppm), multidrug (8.90 ppm) and quinolone (3.60 ppm) (Fig. 1a). The total ARG abundance decreased from 125.97 ppm in mesophilic sludge (day 0) to 50.65 ppm in thermophilic sludge (day 57). The abundances of the three dominant resistance genes decreased to 11.73, 5.81 and 5.99 ppm, respectively, in thermophilic sludge, and chloramphenicol, multidrug, quinolone, fosmidomycin and trimethoprim ARGs also decreased by close to or above 50%. Beta-lactam and bacitracin ARGs were only slightly reduced by 1.83% and 9.92%, respectively, while aminoglycoside resistance genes slightly increased from 12.73 ppm to 13.39 ppm after the

Fig. 1. Change in resistome of the digested sludge before and after temperature increase. (a) Abundance and percentage of ARG types in mesophilic anaerobic digestion sludge (Meso-d0) and thermophilic anaerobic digestion sludge (Thermo-d57). (b) Abundance of select ARG subtypes whose sum abundance in mesophilic and thermophilic sludge >1 ppm.

hike of temperature, becoming the most dominant resistance gene type in thermophilic sludge (Fig. 1a). Though the polymyxin and vancomycin ARGs were enriched by 21.23% and 182.88%, respectively, their abundances were very low (<0.2 ppm).

A total of 125 ARG subtypes were detected in the two samples, and the abundances of 37 ones with their individual sum in mesophilic and thermophilic sludge >1 ppm are compared in Fig. 1b. Among the 37 subtypes, 31 were reduced after temperature increase. *sul1*, the most dominant subtype in mesophilic sludge, several *tet* genes (*tet(G)*, *tet(M)*, *tet(A)*, *tet(C)* and *tet(36)*), chloramphenicol efflux pump gene *cml_e3* and erythromycin esterase gene *ere(A)* decreased by 42.90–97.15%. However, six ARG subtypes (*aadE*, *aadA*, *aacA4*, *mef(A)*, *bl2d_oxa10* and *catb3*) in digested sludge were enriched by 2.86–130.92% after the temperature increase.

Search in INTEGRALL database revealed that a total of 230 types of known integron related genes were matched with 192 in mesophilic sludge and 170 in thermophilic sludge, which all belonged to *intI1*. As shown in Fig. 2 and S4, the abundance of integrons decreased from 123.45 to 70.48 ppm after temperature increase.

Alignment against the ISfinder database showed that a total of 1045 types of known ISs, which can be further classified into 25 IS families, were detected with 814 in mesophilic sludge and 782 in thermophilic sludge. After temperature increase, their sum abundance decreased from 528.73 to 391.36 ppm (Fig. 2). IS256 (134.15 ppm), IS3 (110.73 ppm) and Tn3 (100.76 ppm) were the dominant IS families in mesophilic sludge, accounting for over 65% of the total IS abundance. After the temperature hike, the abundances of IS256 and IS3 decreased to 42.13 and 48.77 ppm, respectively, while Tn3 slightly increased (4.01%) (Fig. S5).

Totally 1554 types of plasmid related genes were identified by searching the NCBI Plasmid Genome Database, with 1298 in

mesophilic sludge and 1282 in thermophilic sludge, and the total abundance decreased from 2984.82 to 2347.47 ppm after temperature increase (Fig. 2). The dominant plasmids in mesophilic sludge were pTM3, plasmid CRM-0020, plasmid WSH-002_p1, pPRO2, pECD227_112 and pGBRO01. The abundances of pPRO2, pECD227_112 and pGBRO01 decreased by 54.29, 69.93 and 99.83%, respectively, while those of pTM3 and plasmid CRM-0020 remained almost unchanged, and plasmid WSH-002_p1 increased from 80.74 to 139.54 ppm in thermophilic sludge (Fig. S6).

To investigate the distribution of ARGs on plasmids, the presence of ARG sequences bearing contigs on plasmids were analyzed (Tables S5 and S6). A total of 41 contigs associated with 20 resistance subtypes were found to be on 25 plasmids in mesophilic sludge (Table S6). Among the 20 ARG subtypes, *aadB*, *cml_e1*, *ereA*, *sul1*, *tet(33)*, *tet(G)*, *tet(M)* and *tet(W)* were simultaneously carried by two or three different plasmids. Moreover, plasmid p1 from *Klebsiella pneumoniae* JM45 and p3 from *Enterococcus faecium* Aus0085 contained two resistance subtypes, while pECD227_112 contained three ARGs (*sul1*, *tet(A)* and *aadA*) (Table S5). By comparison, only 14 contigs associated with 11 ARGs were identified on 13 plasmids on day 57. Among the resistance subtypes detected in thermophilic sludge, *ereA*, *sul2* and *tet(C)* were located on two different plasmids, and only pNDMCFuy from *Citrobacter* simultaneously contained two resistance subtypes (*tet(C)* and *bl2d_oxa10*) (Table S6).

3.2. Changes in 24 ARGs and *intI1* over the sludge transformation period

Twenty-four ARGs belonging to three ARG types and *intI1*, which were detected in sludge resistome and mobilome, were further quantified by qPCR over the sludge transformation period. As

Fig. 2. Change in mobilome of the digested sludge before and after temperature increase.

Fig. 3. Total abundances of tetracycline, macrolide and aminoglycoside resistance genes in sewage sludge, mesophilic sludge (Day 0) and thermophilic sludge (Day 57) quantified by qPCR.

shown in Fig. 3, the feed sludge exhibited high total ARG relative abundance (2.40×10^{-2} ; relative to 16S rRNA), with total abundances of tetracycline, macrolide and aminoglycoside resistance genes being 1.60×10^{-2} , 4.61×10^{-3} and 3.40×10^{-3} , respectively. The levels of the first two types of ARGs were in accordance with those reported in previous studies (Liu et al., 2012, 2014), while the relative abundances of the eight aminoglycoside resistance genes in

sewage sludge were quantified for the first time. As shown in Table S3, the relative abundances of the individual ARGs in the feed sludge ranged from 10^{-6} to 10^{-3} , with *tet(A)* ($6.59 \times 10^{-3} \pm 5.71 \times 10^{-4}$) being the most abundant and *aphA1* ($6.48 \times 10^{-6} \pm 3.98 \times 10^{-7}$), *Mph(B)* ($6.20 \times 10^{-6} \pm 9.42 \times 10^{-8}$) and *mef(A)* ($4.39 \times 10^{-6} \pm 1.55 \times 10^{-7}$) being the rarest.

The sum of the relative abundances of the 24 ARGs in the feed sludge decreased by 38.82% and 64.99% after mesophilic and thermophilic AD treatment, respectively. After temperature increase, the reduction rates of tetracycline and macrolide resistance genes increased from 46.53% to 69.84% and 1.98% to 89.45%, respectively, while that of aminoglycoside resistance genes decreased from 52.46% to 9.05% (Fig. 3). The changes in individual ARGs over the transition period are shown in Figs. S1–S3. According to their profiles on day 0 (mesophilic digestion) and day 57 (thermophilic digestion), the 24 detected ARGs could be divided into three categories (Table 1). Except for *tet(L)* in mesophilic AD ($P = 0.054$) and *aadA* in thermophilic AD ($P = 0.087$), significantly reduced ARGs under both AD conditions ($P < 0.05$) included *tet(A)*, *tet(C)*, *tet(G)*, *tet(L)*, *tet(O)*, *tet(M)*, *tet(Q)*, *tet(W)*, *tet(X)*, *erm(B)*, *erm(T)*, *erm(X)*, *aac(3)-II*, *aacA4*, *aadA*, *aadB*, *aphA1*, *strA*, and *strB*; ARGs that significantly increased in mesophilic AD ($P < 0.05$), but decreased in thermophilic AD ($P < 0.05$) included *ermF* and *ereA*; ARGs that decreased in mesophilic AD ($P < 0.01$), but increased in thermophilic AD ($P < 0.05$) included *mph(B)*, *mef(A)*, and *aadE*.

Feed sludge also had a high level ($7.89 \times 10^{-2} \pm 6.62 \times 10^{-3}$) of *intI1*, which was in accordance with previous studies (Diehl and LaPara, 2010; Ma et al., 2011). Its abundance decreased by 47.51% ($P < 0.05$) and 71.90% ($P < 0.01$) after mesophilic and thermophilic AD, respectively (Table 1). As shown in Table S7, among the 24 ARGs detected, only *tet(A)*, *tet(G)*, and *tet(Q)* displayed a positive correlation with *intI1* ($P < 0.05$) during the transition period.

Table 1

Reduction of 24 ARGs and *intI1* in sewage sludge by mesophilic (Day 0) and thermophilic (Day 57) AD detected by qPCR.

Resistance genes		Removal rate (%) ^a	
		Mesophilic AD	Thermophilic AD
Tetracyclines	<i>tet(A)</i>	40.82	79.36
	<i>tet(C)</i>	38.70	51.98
	<i>tet(G)</i>	39.54	55.67
	<i>tet(L)</i>	40.80	65.92
	<i>tet(Q)</i>	88.99	86.45
	<i>tet(O)</i>	21.71	67.58
	<i>tet(M)</i>	47.41	65.71
	<i>tet(W)</i>	82.50	81.14
	<i>tet(X)</i>	68.56	88.19
	<i>erm(B)</i>	90.03	95.75
Macrolides	<i>erm(F)</i>	−104.99	93.06
	<i>erm(T)</i>	93.49	89.48
	<i>erm(X)</i>	88.29	84.82
	<i>mef(A)</i>	60.21	−20.93
	<i>ereA</i>	−61.37	42.97
	<i>Mph(B)</i>	52.23	−40.56
	<i>aac(3)-II</i>	73.17	55.97
Aminoglycosides	<i>aacA4</i>	53.95	33.42
	<i>aadA</i>	38.18	19.07
	<i>aadB</i>	65.87	60.13
	<i>aadE</i>	78.10	−314.31
	<i>aphA1</i>	71.35	52.14
	<i>strA</i>	55.88	44.14
	<i>strB</i>	57.44	40.27
	<i>intI1</i>	47.51	71.90

^a The removal rate (%) is based on the difference in the individual resistance genes from the AD sludge vs. the sewage sludge (untreated).

3.3. Co-occurrence between 24 ARGs and bacterial taxa

After the increase in temperature, both the abundance and diversity of the bacterial population in the digested sludge were decreased (Tian et al., 2015). To find the possible host information for individual ARGs, the co-occurrence patterns between the 24 ARGs and bacterial taxa during the transition period were investigated using network analysis (Fig. 4), based on the hypothesis that the significantly similar abundance trends among different samples was because of some specific bacterial taxa carrying some specific ARGs (Forsberg et al., 2014; Li et al., 2015). The detailed co-occurrence between ARGs and bacterial taxa is summarized in Table S8. As shown in Fig. 4 and Table S8, the total abundance of 18 bacterial genera, which were suggested as the possible hosts of 13 ARGs through network analysis, decreased from 23.27% in mesophilic sludge to 11.92% in thermophilic sludge. *Bacteroides* and *Exilispira* might be the hosts of tetracycline resistance gene *tet(X)*, and *Levilinea* might contain another two tetracycline resistance genes (*tet(A)* and *tet(G)*). *Ruminococcus*, *Ottowia*, *Gordonibacter* and

Lautropia were found to be the hosts of four aminoglycoside resistance subtypes (*strB*, *strA*, *aphA1* and *aadB*), while another aminoglycoside nucleotidyltransferase gene *aadE* was mainly carried by *Syntrophaceticus* and *Haloplasma*. *Diaphorobacter* and *Dechloromonas* took along the MLS efflux gene (*mef(A)*), and EM3, a typical thermophilic genus (Hugenholtz et al., 1998), mainly carried the MLS phosphorylase gene (*Mph(B)*).

4. Discussion

The purpose of this study was to explore the mechanisms behind the reduction of ARGs during mesophilic and thermophilic AD processes. Different from previous studies that compared steady-state reactors operated in parallel (Ghosh et al., 2009a; Diehl and LaPara, 2010; Ma et al., 2011; Zhang et al., 2015), this study focused on the dynamic transition of an anaerobic digester from mesophilic steady-state to thermophilic one to reveal how resistance-related items (resistomes, mobilomes and bacterial hosts) in digested sludge respond to the increase of digestion

Fig. 4. Network analysis revealing the co-occurrence patterns between ARG subtypes and bacterial taxa during the transition period.

temperature by jointly using metagenomics, qPCR and 454-pyrosequencing. Previous study has demonstrated that the outputs from the qPCR and metagenomic analyses support the same results for detecting ARGs no matter in their total quantity or individual ARG abundance (Aydin et al., 2015). In this study, both metagenomic and qPCR analyses showed that thermophilic sludge held a lower overall ARG abundance than that of the mesophilic one (Figs. 1 and 3). Using parallel bench-scale reactors, Zhang et al. (2015) found that no measurable difference in the abundance of total ARGs in either thermophilic or mesophilic sludge treating the secondary sludge, which was not in accordance with this study. One of the possible reasons for the inconsistency might be the difference in feed sludge. Regarding individual ARGs, thermophilic AD showed better performance in the reduction of *acrB*, *tet(A)*, *tet(L)*, *tet(O)*, *tet(M)*, *tet(X)*, *sul1*, *erm(B)*, *ermF* and *ereA*, which was in accordance with previous studies (Ghosh et al., 2009a; Diehl and LaPara, 2010; Ma et al., 2011; Zhang et al., 2015). Mesophilic AD showed better performance in the reduction of *aac(3)-II*, *aacA4*, *aadA*, *aadB*, *aadE*, *aphA1*, *strA*, *strB*, *meff(A)*, *mph(B)* and *qnrS*, and the results of *aadA*, *strA* and *qnrS* match the observation by Zhang et al. (2015). The above results indicated that ARG subtypes exhibited varied responses to mesophilic and thermophilic treatment (Fig. 1b and Table 1), which was also observed by Ma et al. (2011) and Zhang et al. (2015).

The reduction of ARGs during AD has been speculated to be associated with the blockage of their horizontal transfer pathways (Diehl and LaPara, 2010; Ma et al., 2011). Metagenomic analysis demonstrated that the abundance of mobilome including integrons, insert sequences and plasmids in thermophilic sludge was lower than that in mesophilic sludge (Fig. 2), suggesting lower horizontal transfer potential for ARGs under thermophilic conditions. Integrons, usually located in transposons or plasmids (Guerin et al., 2009), are important genetic elements for ARG recombination, and can acquire and express exogenous ARGs embedded within gene cassettes to accelerate their transfer (Stokes and Hall, 1989). As the most abundant integron in environmental bacteria (Stalder et al., 2014), *intI1* has been suggested as a proxy for antibiotic pollution (Gillings et al., 2015; Aydin et al., 2015). In this study, all integron related sequences in sludge metagenomes belonged to *intI1* and their abundances moderately decreased after temperature increase. Two efflux (*tet(A)* and *tet(G)*) and one ribosomal protection (*tet(Q)*) tetracycline resistance genes were positively correlated with *intI1*, which was in accordance with the previous study on an oxytetracycline production wastewater treatment system, expect for *tet(Q)* (Liu et al., 2012). Neither *tet(A)* nor *tet(G)* have been detected as gene cassettes of *intI1* (Partridge et al., 2009). However, *tet(A)* has been found to coexist with *intI1* in conjugative plasmids (L'Abée-Lund and Sorum, 2001; Agerso and Sandvang, 2005), whereby they can be easily co-transferred into recipients, while *tet(G)* has been found on the pattern 3 insertion sequence common region, a newly highlighted complex integron element usually linked to *intI1* (Toleman et al., 2006).

Both ISs and plasmids are bacterial genetic elements responsible for the transfer of ARGs between different bacterial cells (Aminov, 2011). Compared with integrons, the two mobile elements had high diversity in the mesophilic and thermophilic sludge. After temperature increase, their abundances also decreased, and their compositions showed evident shifts (Figs. S5, S6 and S7), which might be driven by the succession of anaerobic microbial communities. For example, ISEnc1 of the mesophilic hydrolytic-fermentative bacterium *Enterococcus* (Chen et al., 2006) decreased together with its host, while IScth5 and ISPeth3, which belong to thermophilic hydrolytic-fermentative *Clostridium thermocellum* (Wilson et al., 2013) and thermophilic syntrophic propionate-oxidizing bacterium *Pelotomaculum thermopropionicum*

(Imachi et al., 2002), respectively, were detected with high abundances in the thermophilic sludge (Fig. S5). Similarly, plasmids p5482, p04 and pMUR050 found in mesophilic bacterial genera *Bacteroides*, *Bacillus*, and *Escherichia* (Xu et al., 2003; Gonzalez-Zorn et al., 2005), respectively, were decreased, while plasmid pTLIE01 originating from *Thermovirga* (Goeker et al., 2012), a thermophilic amino-acid-degrading bacterium (Dahle and Birkeland, 2006), was enriched more than 100-fold after the temperature increase.

The shift in the diversities of ISs and plasmids might impact the transferability of ARGs. Through coupling profile analysis between ARGs and plasmids, the dominant driver of DNA exchange in prokaryotes (Sorensen et al., 2005), a greater number of ARGs were found located in plasmids in the mesophilic sludge than those in the thermophilic sludge (Tables S5 and S6). The coupling of several resistance subtypes, including *tet(X)*, *tet(G)*, *tet(33)*, *erm(G)* and *cml_e1*, whose abundances decreased over the transition period, with plasmids also lost after temperature increase, suggesting the decreased transferability for these ARGs under thermophilic conditions. While for the enriched resistance gene *aacA4*, its coupling to plasmid occurred in the thermophilic sludge, suggesting higher transfer possibility of this gene through plasmid conjugation under thermophilic conditions. Thus, the lower availability of mobilomes including integrons, insert sequences and plasmids in thermophilic sludge might be partly responsible for the decreased abundance of ARGs.

On the other hand, it is known that the mesophilic anaerobic bacterial community is very different from the thermophilic one, which is characterized by a relatively low diversity (Sasak et al., 2011; Tian et al., 2015). Such a shift in bacterial community during the transition period might directly impact the vertical transfer of ARGs. Through network analysis, it was possible to assign the possible hosts for many targeted ARGs (Li et al., 2015; Ju et al., 2016). For example, sulfate-reducing *Desulfobulbus*, chlorate-reducing *Dechloromonas* and thermophilic fermentative EM3 were determined to be the possible hosts of *tet(L)*, *meff(A)* and *Mph(B)*, respectively. Total abundance of the 18 bacterial genera, which were suggested as the possible hosts for 13 ARGs, was decreased by close to 50% after temperature increase, suggesting that decreased vertical transferability of resistance genes might also be an important mechanism of resistome reduction.

The hosts of *tet(X)* and *aadE* have been verified in previous studies (Table S8). Although recently also found in aerobic *Sphingobacterium* sp. (Ghosh et al., 2009b) and some *Enterobacteriaceae* and *Pseudomonadaceae* strains (Leski et al., 2013), the host diversity of *tet(X)* is relatively low (Chopra and Roberts, 2001), being mainly carried by *Bacteroides* (Guiney et al., 1984; Bartha et al., 2011; de Vries et al., 2011; Forslund et al., 2013). As described previously (Tian et al., 2015), *Bacteroides* was the dominant fermentative bacteria in mesophilic sludge, and experienced fast decline after temperature increase because all species of this genus are mesophilic (Garrity et al., 2004). At the same time, *tet(X)* has not yet been proved to be horizontally transferred to other strains (Ghosh et al., 2009b), in spite of the fact that it has been linked with a variety of mobile genetic elements (Guiney et al., 1984; Robillard et al., 1985; Shoemaker et al., 1986; Ghosh et al., 2009b). Therefore, the high reduction efficiency of *tet(X)* during thermophilic AD could be attributed to the elimination of its bacterial host by the high temperature. It is interesting that *aadE* and *Haloplasma* rebounded after the temperature increase (Table S8 and Fig. S3). *aadE* is reported to be harbored by *Haloplasma* (Forslund et al., 2013), whose only type species, *Haloplasma contractile*, is mesophilic (Antunes et al., 2008). It is possible that some unknown thermophilic species in *Haloplasma* might be responsible for the observed relevance between *aadE* and *Haloplasma*. In addition to the *aadE* gene, some aminoglycoside resistance genes also increased after the temperature

hike (Table 1, Fig. S3), and significant correlations existed between the eight aminoglycoside resistance genes themselves (Fig. 4, Table S9), which might be attributed to the coexistence of aminoglycoside resistance genes in bacterial genomes (Karczmarczyk et al., 2011; Cain and Hall, 2012). Thus, co-selection might play an important role in their dissemination during the transition period.

It should be noted that the suggested hosts shown in Table S8 may not be consistent with their reported hosts, which was also observed by Li et al. (2015) using the same network analysis method. The inconsistency might be due to the fact that the reported hosts of ARGs have mainly been isolated from clinical settings, which differ from environmental bacteria (Sommer et al., 2009; Forsberg et al., 2014). To further validate the co-occurrence relationships revealed by network analysis, additional research is required to isolate bacterial hosts of ARGs from various environments. Furthermore, new techniques, such as functional metagenomics that allow direct linkage of detected ARGs with their potential hosts (Forsberg et al., 2014; Udikovic-Kolic et al., 2014), could be employed to better understand the role of vertical gene transfer in the dissemination of ARGs. On the other hand, metagenomic sequencing or qPCR used for the detection of ARGs cannot distinguish ARGs from live or dead cells in digested sludge, which may partially distort the temporal and spatial patterns of the detected ARGs. Also, a greater number of sludge samples for metagenomic analysis are needed to validate the result of this study.

5. Conclusions

This study showed a decrease in the total abundance of ARGs in anaerobic sludge treating sewage sludge after the increase of digestion temperature from mesophilic to thermophilic. Together with the decrease of resistome, the mobilome and potential bacterial hosts of ARGs in anaerobic digested sludge were also decreased after temperature increase, suggesting that the lower level of ARGs in the thermophilic sludge might be associated with the decrease in their horizontal and vertical transfer abilities. Based on these results, sludge treatment technologies like thermophilic AD, which can effectively remove the mobile genetic elements and bacterial hosts of ARGs, are recommended for the treatment of sewage sludge in full-scale WWTPs to limit the release of ARGs into the environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2016.04.031>.

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